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(54) A METHOD OF PREPARING A VARIANT OF A LIPOLYTIC ENZYME

METHODE ZUR HERSTELLUNG EINER VARIANTE EINES LIPOLYTISCHEN ENZYMES

PROCEDE POUR PREPARER UN VARIANT D'UNE ENZYME LIPOLYTIQUE

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EP 0 746 618 B1

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Description

FIELD OF THE INVENTION

[0001] The present invention relates to a method of preparing a variant of a parent lipolytic enzyme and to variants prepared by the method. Furthermore, the invention relates to a DNA construct encoding a variant of the invention, an expression vector and host cell comprising the DNA construct and a detergent additive or a detergent composition comprising a variant.

BACKGROUND OF THE INVENTION

[0002] For a number of years lipolytic enzymes have been used as detergent enzymes, i.e. to remove lipid or fatty stains from clothes and other textiles.

[0003] For instance, various microbial lipases have been suggested as detergent enzymes. Examples of such lipases include a *Humicola lanuginosa* lipase, e.g. described in EP 258 068 and EP 305 216, a *Rhizomucor miehei* lipase, e. g. as described in EP 238 023, a *Candida* lipase, such as a *C. antarctica* lipase, e.g. the *C. antarctica* lipase A or B described in EP 214 761, a *Pseudomonas* lipase such as a *P. alcaligenes* and *P. pseudoalcaligenes* lipase, e.g. as described in EP 218 272, a *P. cepacia* lipase, e.g. as described in EP 331 376, a *Bacillus* lipase, e.g. a *B. subtilis* lipase (Dartois et al., 1993), a *B. stearothermophilus* lipase (JP 64/744992) and a *B. pumilus* lipase (EP 91 00664).

[0004] Furthermore, a number of cloned lipases have been described, including the *Penicillium camembertii* lipase described by Yamaguchi, S. et al., 1991, the *Geotricum candidum* lipase (Schimada, Y. et al., 1989), and various *Rhizopus* lipases such as a *R. delemar* lipase (Hass, M.J et al., 1991), a *R. niveus* lipase (Kugimiya, W. 1992), and a *R. oryzae* lipase.

[0005] Other types of lipolytic enzymes having been suggested as detergent enzymes include cutinases, e.g. derived from *Pseudomonas mendocina* as described in WO 88/09367, or a cutinase derived from *Fusarium solani pisi* (e.g. described in WO 90/09446).

[0006] In recent years attempts have been made to prepare lipase variants having improved properties for detergent purposes. For instance, WO 92/05249 discloses lipase variants with improved properties, in which certain characteristics of wild-type lipase enzymes have been changed by specific, i.e. site-directed modifications of their amino acid sequences. More specifically, lipase variants are described, in which one or more amino acid residues of the so-called lipid contact zone of the parent lipase has been modified.

[0007] PCT/DK93/00225 describes lipase variants with improved properties, in which an amino acid residue occupying a critical position of the lipase has been modified.

[0008] EP 407 225 discloses lipase variants with improved resistance towards proteolytic enzymes, which have been prepared by specifically defined amino acid modifications.

[0009] EP 260 105 describe hydrolases in which an amino acid residue within 15 Å from the active site has been substituted.

[0010] All of the above mentioned lipase variants have been constructed by use of site-directed mutagenesis resulting in a modification of specific amino acid residues which have been chosen either on the basis of their type or on the basis of their location in the secondary or tertiary structure of the parent lipase.

[0011] An alternative approach for constructing mutants or variants of a given protein has been based on random mutagenesis. For instance, US 4,898,331 and WO 93/01285 disclose such techniques.

[0012] A need exists for novel lipolytic enzymes having improved washing and/or dishwashing properties, and the object of the present invention is to prepare such enzymes.

BRIEF DISCLOSURE OF THE INVENTION

[0013] The present inventors have now developed a novel method of preparing variants of lipolytic enzymes having improved washing and/or dishwashing performance as compared to their parent enzymes. The method is based on random or localized random mutagenesis of DNA sequences encoding a lipolytic enzyme.

[0014] More specifically, in a first aspect the invention relates to a method of preparing a variant of a parent lipolytic enzyme, which method comprises

(a) subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis,

(b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

(c) screening for host cells expressing a mutated lipolytic enzyme which has a decreased dependance to calcium

and optionally an improved tolerance towards a detergent or one or more detergent components as compared to the parent lipolytic enzyme.

[0015] In the present context, the term "lipolytic enzyme" is intended to indicate an enzyme exhibiting a lipid degrading capability, such as a capability of degrading a triglycerid or a phospholipid. The lipolytic enzyme may, e.g., be a lipase, a phospholipase, an esterase or a cutinase.

[0016] The term "random mutagenesis" is intended to be understood in a conventional manner, i.e. to indicate an introduction of one or more mutations at random positions of the parent enzyme (i.e. as opposed to site-specific mutagenesis). The random mutations are typically introduced by exposing a large number of copies of the DNA sequence to be modified to a mutagen and then screening for the presence of variants. Suitable techniques for introducing random mutations are discussed in detail below.

[0017] The screening criteria of step c) are considered to be of particular use in identifying variants of parent lipolytic enzymes having improved washing and/or dishwashing performance as compared to their parent enzymes.

[0018] In the present context, the term "decreased dependance to calcium" is intended to mean that the mutated lipolytic enzyme requires lower amounts of calcium for exhibiting the same degree of activity as the parent enzyme when tested under similar conditions. Preferably, the mutated lipolytic enzyme of the invention is substantially independant of the presence of calcium for exhibiting enzymatic activity.

[0019] The term "improved tolerance towards a detergent or detergent component" is intended to mean that the mutated lipolytic enzyme is active at higher concentrations of the detergent or detergent component than the parent lipolytic enzyme.

[0020] In the present context the term "detergent" is intended to indicate a mixture of detergent ingredients normally used for washing or dishwashing. Analogously, a "detergent component" is intended to indicate a component or ingredient normally found in detergent or dishwashing compositions, examples of which are given in the following description.

[0021] It will be understood that the variant prepared by the method of the invention in addition to the decreased dependency to calcium and optionally improved tolerance towards a detergent or one or more detergent components exhibits lipolytic activity preferably of a magnitude comparable to or exceeding that of the parent lipolytic enzyme, when tested under washing and/or dishwashing conditions.

[0022] The screening criteria defined in step c) of the method of the invention may be determined by any suitable methods known in the art. A particular suitable assay developed for the present purpose is described in the Materials and Methods section below.

[0023] In final aspects the invention relates to a variant of a lipolytic enzyme and the use of said variant as a detergent enzyme, in particular for washing or dishwashing, and to a detergent additive and a detergent composition comprising the variant.

DETAILED DISCLOSURE OF THE INVENTION

Cloning a DNA sequence encoding a parent lipolytic enzyme

[0024] The DNA sequence encoding a parent lipolytic enzyme to be subjected to random mutagenesis in accordance with the present invention may be isolated from any cell or microorganism producing the parent enzyme in question by use of methods known in the art.

[0025] For instance, the DNA sequence may be isolated by establishing a cDNA or genomic library from an organism expected to harbour the sequence, and screening for positive clones by conventional procedures. Examples of such procedures are hybridization to oligonucleotide probes prepared on the basis of the amino acid or DNA sequence of the parent enzyme (if sequence information is available) or of a related lipolytic enzyme (if sequence information as to the parent enzyme is not available) in accordance with standard techniques (cf. Sambrook et al., 1989), and/or selection for clones expressing lipolytic, such as lipase activity, and/or selection for clones producing a protein which is reactive with an antibody raised against a parent lipolytic enzyme.

[0026] A preferred method of isolating a DNA sequence encoding a parent lipolytic enzyme to be modified in accordance with the invention from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of DNA or amino acid sequence of the parent enzyme. For instance, the PCR may be carried out using the techniques described in US Patent No. 4,683,202 or by R.K. Saiki et al. (1988).

[0027] Alternatively, the DNA sequence encoding the parent enzyme may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and Caruthers (1981), or the method described by Matthes et al. (1984). According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

[0028] Finally, the DNA sequence encoding the parent enzyme may be prepared from DNA of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic,

genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence encoding the parent enzyme, in accordance with standard techniques.

Random mutagenesis

[0029] The random mutagenesis of the DNA sequence encoding the parent lipolytic enzyme to be performed in accordance with step a) of the method of the invention may conveniently be performed by use of any method known in the art.

[0030] For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

[0031] The mutagenizing agent may, e.g., be one which induces transitions, transversions, inversions, scrambling, deletions, and/or insertions.

[0032] Examples of a physical or chemical mutagenizing agent suitable for the present purpose includes ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), o-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

[0033] When such agents are used the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

[0034] When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions wanted to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the lipolytic enzyme by any published technique using e.g. PCR, LCR or any DNA polymerase and ligase.

[0035] When PCR generated mutagenesis is used either a chemically treated or non-treated gene encoding a parent lipolytic enzyme is subjected to PCR under conditions that increases the mis-incorporation of nucleotides (Deshler 1992, Leung et al. 1989).

[0036] A mutator strain of *E. coli* (Fowler et al. 1974), *S. cereviciae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the lipolytic enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid from the mutator strain. The mutated plasmid may subsequently be transformed into the expression organism.

[0037] The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent lipolytic enzyme. Alternatively, the DNA sequence may be present on a suitable vector such as a plasmid or a bacteriophage, which as such may.be incubated with or otherwise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

[0038] In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step (b) or the screening step (c) being performed. Such amplification may be performed in accordance with methods known in the art, the presently preferred method being PCR generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

[0039] Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are given below. The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

[0040] It will be understood that the screening criteria mentioned in step (c) above have been carefully selected. Thus, without being limited to any theory the screening for a decreased dependency to calcium is believed to result in variants having an over-all improved performance in that the requirement for calcium may be considered a limiting factor for optimal activity, in particular under conditions where only low amounts of free calcium ions are present. In connection with detergent lipases the free calcium ions required are normally provided from the washing water and thus, the lipolytic activity is dependent on the calcium content of the water.

[0041] The detergent or detergent component towards which the variant has improved tolerance may be of any type, e.g. as further described below. Preferably, the detergent component is a non-ionic, anionic, kationic, zwitterionic or amphoteric surfactant. Examples of non-ionic surfactants include an alcohol ethoxylate, examples of anionic sur-

factants include LAS, alkyl sulphate, alcohol ethoxy sulphate and the like.

[0042] In particular, it is contemplated that an improved tolerance towards a non-ionic surfactant alcohol ethoxylate, a commercially available example of which is Dobanol®, may be indicative of improved wash performance.

[0043] The screening of step (c) is conveniently performed by use of a filter assay based on the following principle:

[0044] A microorganism capable of expressing the mutated lipolytic enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the microorganisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

[0045] The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The topfilter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore™. The filter may be pretreated with any of the conditions to be used for screening or may be treated during the detection of enzymatic activity.

[0046] The enzymatic activity may be detected by a dye, flourescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

[0047] The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

[0048] Lipase activity may be detected by Brilliant green, Rhodamine B or Sudan Black in combination with a lipid e.g. olive oil or lard. The screening criteria for identifying variants of parent lipolytic enzymes having improved washing performance may be e.g. EGTA, EDTA, non-ionic or anionic tensides, alkaline pH, or any detergent composition in combination with one of the above detectors of enzymatic activity.

[0049] It will be understood that the screening criteria used in the filter assay of the invention may be chosen so as to comply with the desired properties or uses of the enzymes to be screened. For instance, in a screening for lipases of particular use in the paper and pulp industry, it may be relevant to screen for an acid lipase having an increased temperature stability. This may be performed by using a buffer with acidic pH (e.g. pH 4) and/or incubate under higher temperature before or under the assay.

[0050] The host cells produced in step (c) may be subjected to further rounds of mutagenesis as defined in steps (a)-(c) above, conveniently by using more stringent selection criteria than employed in a previous mutagenesis treatment.

[0051] The host cells selected for in step (c) may be used directly for the production of the variant of the lipolytic enzyme. Alternatively, DNA encoding the variant may be isolated from the host cell and inserted into another suitable host cell, conveniently by use of the procedure described below in the section entitled "Expression of a variant of the invention", in which suitable host cells are also listed.

Localized random mutagenesis

[0052] In accordance with the invention the random mutagenesis may advantageously be located to a part of the parent lipolytic enzyme in question. This may, e.g., be advantageous when a certain region of the enzyme has been identified to be of particular importance for a given property of the enzyme, and which, when modified, is expected to result in a variant having improved properties. Such region may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

[0053] The localized random mutagenesis is conveniently performed by use of PCR generated mutagenesis techniques as described above or any other suitable technique known in the art.

[0054] Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e. g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

The parent lipolytic enzyme

[0055] The parent lipolytic enzyme to be modified in accordance with the invention may be any enzyme which has lipolytic activity as defined above. Examples of lipolytic enzymes includes a lipase, an esterase, a cutinase and a phospholipase.

[0056] Preferably, the parent lipolytic enzyme is modified by localized random mutagenesis performed on a part of the DNA sequence encoding a lipid contact zone or a part of said zone.

[0057] All lipases crystalized until now have been found to comprise at least one surface loop structure (also termed a lid or a flap) which covers the active site when the lipase is in inactive form (an example of such a lipase is described

by Brady et al., 1990). When the lipase is activated, the loop structure is shifted to expose the active site residues, and a hydrophobic surface is created surrounding the active site Ser, which has an increased surface hydrophobicity and which interacts with the lipid substrate at or during hydrolysis. This activation is termed interfacial activation and is further discussed by Tilbeurgh et al. (1993).

[0058] For the present purpose, the surface created upon activation is termed the "lipid contact zone", intended to include amino acid residues located within or forming part of this surface, optionally in the form of loop structures. These residues may participate in lipase interaction with the substrate at or during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface.

[0059] The lipid contact zone contains a binding area for the lipid substrate which is the part of the lipid contact zone to which the single lipid substrate molecule binds before hydrolysis. This binding area again contains an acyl-binding hydrophobic cleft and a so-called hydrolysis pocket, which is situated around the active site Ser, and in which the hydrolysis of the lipid substrate is believed to take place. In all lipases known today the lipid contact zone is easily recognized, e.g. from a three-dimensional structure of the lipase created by suitable computer programs. The conformation of an inactive and activated lipase, respectively, is shown in Figs. 1 and 2 of WO 92/05249.

[0060] The lipid contact zone of the *Humicola lanuginosa* lipase discussed in detail in the present application is defined by amino acid residues 21-25, 36-38, 56-62, 81-98, 110-116, 144-147, 172-174, 199-213 and 248-269. These residues have been identified on the basis of computer model simulations of the interaction between the lipase and a lipid substrate.

[0061] The lipid contact zone of other lipolytic enzymes is defined by

a) calculating the hydrophobic vector of the 3-D molecular structure,
b) making a cut perpendicular to the vector through the Cα-atom of the second amino acid residue after the active site serine in the linear sequence, and
c) including all residues with at least one atom on that side of the cut to which the vector points, and
d) selecting from those residues, those which have at least one atom within 5 Ångström of the surface of the protein (in case of a lipase in either its open or closed form).

[0062] The hydrophobic vector is calculated from 'the protein structure, in case of a lipase either the open or closed form, by summing up all residue vectors for residues having a surface accessibility (Lee, B. and Richards, F.M. 1971. Mol. Biol. **55**:379-400) of at least 10%. The starting point of the residue vector is defined as the Cα-atom of the residue and its direction is through the mass center of the sidechain. The magnitude of each residue vector is defined as the residues relative transfer free energy.

[0063] The surface accessibility of each residue is calculated using the Connolly program.

[0064] Preferably, the localized random mutagenesis is performed on a part of the DNA sequence encoding a lid region and/or a hydrophobic cleft of the parent lipase, or a part of said lid region and/or hydrophobic cleft.

[0065] The parent lipolytic enzyme to be modified in accordance with the invention may be of any origin. Thus, the enzyme may be of mammalian, plant, vertebrate or any other region. However, it is presently preferred that the enzyme is of microbial origin in that a number of microbial strains have been found to produce enzymes of particular use for detergent purposes.

[0066] More specifically, the DNA sequence parent lipolytic enzyme may be derived from a fungus, i.e. a yeast or a filamentous fungus. For instance, the DNA sequence may be one which is derivable form a strain of a *Humicola* sp., e.g. *H. lanuginosa,* a strain of a *Rhizomucor* sp., *e.g. Rh. miehei,* a strain of a *Rhizopus* sp., a strain of a *Candida* sp., a strain of a *Fusarium* sp., e.g. *F. solani pisi,* a strain of a *Venturia* spp., e.g. *V. inaequalis,* a strain of a *Colletotrichum* spp., e.g. *C. gloeosporioides,* or *C. lagenarium,* or a strain of a *Penicillium* spp., e.g. *P. spinulosum* or *P. camembertii.*

[0067] In the present context, "derivable from" is intended not only to indicate an enzyme produced by a strain of the organism in question, but also an enzyme encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Furthermore, the term is intended to indicate an enzyme which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the enzyme in question.

[0068] Of particular interest as a parent lipolytic enzyme is a lipase derivable from a strain of *H. lanuginosa,* e.g. the *H. lanuginosa* strain DSM 4109, a strain of *Rh. mucor,* or a strain of *C. antarctica.*

[0069] The variant may be a variant of the *H. lanuginosa* lipase, which further comprises addition of one or more amino acid residues to either or both the N- and C-terminal end of the lipase, substitution of one or more amino acid residues at one or more different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the lipase or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence. The modification of the DNA sequence may be performed by site-directed or by random mutagenesis or a combination of these techniques in accordance with well-known procedures.

[0070] The parent lipolytic enzyme to be modifier in accordance with the present invention may be derivable from a bacterium. For instance, the DNA sequence encoding the parent lipolytic enzyme may be derivable from a strain of *Pseudomonas* spp., such as *P. cepacia, P. alcaligenes, P. pseudoalcaligens, P. mendocina* (also termed *P. putida*), *P. syringae, P. aeroginosa* or *P. fragi,* a strain of *Bacillus* spp., e.g. *B. subtilis* or *B. pumilus* or a strain of *Streptomyces sp.,* e.g. *S. scabies.*

[0071] The parent bacterial lipolytic enzyme may be a lipase derived from any of the above-mentioned species, e. g. a *Pseudomonas* lipase as described in EP 218 272, EP 331 376 and EP 407 225, or a cutinase, e.g. as described in WO 88/09367.

Variants of the invention

[0072] For ease of reference specific variants of the invention are described by use of the following nomenclature:

[0073] Original amino acid(s):position(s):substituted amino acid(s)

[0074] According to this nomenclature, for instance the substitution of aspartic acid for valine in position 96 is shown as:

Asp 96 Val or D96V a deletion of aspartic acid in the same position is shown as:

Asp 96 * or D96* and insertion of an additional amino acid residue such as lysine is shown as:

Asp 96 ValLys or D96VK

[0075] Multiple mutations are separated by pluses, i.e.:

Asp 96 Val + Glu 87 Lys or D96V+E87K representing mutations in positions 96 and 87 substituting aspartic acid and glutamic acid for valine and lysine, respectively.

[0076] When one or more alternative amino acid residues may be inserted in a given position it is indicated as

D96V,N or

D96V or D96N

[0077] Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an aspartic acid in position 96 is mentioned, but not specified, it is to be understood that the aspartic acid may be deleted or substituted for any other amino acid, i.e. any one of R,N,A,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V, or a further amino acid residue inserted at that position.

[0078] Preferably, the variant according to the invention comprises at least one of the following mutations K46R, E57G, G61S, S83T, S58F, D62C, T64R, I90F, G91A, N92H, N94I, N94K, L97M, K98I, I100V, D102K, A121V, E129K, D167G, R205K, E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q or T267W.

[0079] These positions have been found or is contemplated to be important for enzymatic activity and/or detergent tolerance. The numbering of the amino acid residues refers to the amino acid sequence of the mature lipase.

[0080] Preferably, the variant according to this aspect of the invention comprises at least one of the following mutations S83T, N94K, A121V, D167G, R205K.

[0081] It will be understood that the present invention encompasses variants of the parent *H. lanuginosa* lipase comprising a combination of two or more of the mutations defined herein, or a combination of one or more of the mutations defined herein with any of the mutations disclosed in WO 92/05249, WO 94/25577 and WO 94/01541.

[0082] In a further aspect the present invention relates to a variant of the *H. lanuginosa* lipase obtainable from DSM 4109 comprising at least one of the following mutations:

N94K+D96A
S83T+N94K+D96N
E87K+D96V
E87K+G91A+D96A
N94K+F95L+D96H
F95C+D96N
E87K+G91A+D96R+I100V
E87K+G91A
S83T+E87K+Q249R
S83T+E87K+W89G+G91A+N94K+D96V
N73D+S85T+E87K+G91A+N94K+D94A
E87K+G91A+L93I+N94K+D96A
D167G+E210V
N73D+E87K+G91A+N94I+D96G
S83T+E87K+G91A+N92H+N94K+D96M
E56R+D57L+V60M+D62N+S83T+D96P+D102E
D57G+N94K+K96L+L97M

E87K+G91A+D96R+I100V+E129K+K237M+I252L+P256T+G263A+L264Q
E56R+D57G+S58F+D62C+T64R+E87G+G91A+F95L+D96P+K98I+K237M
D167G
N73D+E87K+G91A+N94I+D96G
N251W+D254W+T267W
S83T+E87K+G91A+N92H+N94K+D96M
D57G+N94K+D96L+L97M

[0083] These variants have been found to exhibit a decreased resistance to calcium and/or an improved tolerance towards detergent components, such as the non-ionic surfactant alcohol ethoxylate and are, accordingly, considered of particular use for detergent or dishwashing purposes. The variants have been constructed by the method of the invention and subsequently characterized with respect to the mutations having been introduced and are further described in the Examples hereinafter. It will be apparent that an alternative method of constructing these variants would be based on site-directed mutagenesis using suitable oligonucleotide probes. This method is exemplified in Examples 3-6.

Expression of a variant of the invention

[0084] According to the invention, a mutated DNA sequence encoding a variant lipolytic enzyme prepared by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

[0085] The recombinant expression vector carrying the DNA sequence encoding a variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0086] In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli,* the *Streptomyces coelicolor* agarase gene *dagA promoters, the* promoters of the *Bacillus licheniformis* α-amylase gene (*amy*L), e.g. as described in WO 93/10249 the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amy*M), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amy*Q), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

[0087] The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding a variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

[0088] The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

[0089] The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis,* or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

[0090] While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. The parent lipolytic enzyme may in itself comprise a preregion permitting secretion of the expressed enzyme into the culture medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, convenient accomplished by substitution of the DNA sequences encoding the respective preregions.

[0091] The procedures used to ligate the DNA construct of the invention encoding a variant of a parent lipolytic enzyme, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al.

(1989)).

[0092] The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a variant of a parent lipolytic enzyme of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described below in connection with the different types of host cells.

[0093] The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

[0094] Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis,* or *Streptomyces lividans* or *Streptomyces murinus,* or gramnegative bacteria such as *E.coli.* The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se.*

[0095] The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces,* e.g. *Saccharomyces cerevisiae.* The filamentous fungus may advantageously belong to a species of *Aspergillus,* e.g. *Aspergillus oryzae, Aspergillus niger* or *Aspergillus nidulans.* Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se.* A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

[0096] In a yet further aspect, the present invention relates to a method of producing a variant of a parent lipolytic enzyme of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

[0097] The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the variant of a parent lipolytic enzyme of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

[0098] The variant of the invention secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Detergent Additive and Composition for Dishwashing and Washing

[0099] Due to the decreased dependance to calcium and/or improved tolerance towards detergents or detergent components of the variant of the invention, the variant is particularly well suited for implementation into detergent compositions, e.g. detergent compositions intended for performance in the range of pH 7-13, particularly the range of pH 8-11.

Detergent Compositions

[0100] According to the invention, a lipase variant of the invention may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent G_B 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

[0101] The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

[0102] The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cat-

ionic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

[0103] The detergent composition may additionally comprise one or more other enzymes, such as an amylase, a pullulanase, a cutinase, a protease, a cellulase, a peroxidase, an oxidase, (e.g. laccase) and/or another lipase.

[0104] The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

[0105] The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

[0106] The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzene-sulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

[0107] The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

[0108] The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

[0109] The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

[0110] Particular forms of detergent compositions within the scope of the invention include:

(1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2. - 6%
Zeolite (as $NaA1SiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

(2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%

(continued)

Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	is - 21%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

(3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaA1SiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO_4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

(4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 5%
Zeolite (as $NaAlSiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

(5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

(6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaA1SiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

(7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%

(continued)

Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

(8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaA1SiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

(9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaA1SiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	o - 5%

(10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%

(continued)

Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluensulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethylcellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

(11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/ acrylic acid copolymer)	0 - 3%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

(12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alphasulfo fatty acid methyl esters, alkanesulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaA1SiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

(13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

(14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%

(continued)

Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaA1SiO_4$)	10 - 20%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O,2SiO_2$)	.0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

(15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O,2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

(16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

(17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

(18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

(19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

[0111] A lipase variant of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in a detergent composition of the invention, a lipase variant of the invention may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of the lipase variant per liter of wash liquor.

Dishwashing Composition

[0112] The dishwashing detergent composition comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will contain 0-90% of non-ionic surfactant such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

[0113] The detergent composition may contain detergent builder salts of inorganic and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-containing types. The detergent composition usually contains 1-90% of detergent builders.

[0114] Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts especially alkali metal pyrophosphates, orthophosphates, polyphosphates, and phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates and silicates as well as the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites are the best-known representatives.

[0115] Examples of suitable organic builders include the alkali metal, ammonium and substituted ammonium, citrates, succinates, malonates, fatty acid sulphonates, carboxymetoxy succinates, ammonium polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates, polyacetyl carboxylates and polyhydroxsulphonates.

[0116] Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

[0117] The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

[0118] The oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, per-silicates and perphosphates. Preferred activator materials are TAED and glycerol triacetate.

[0119] The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g.propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester.

[0120] The dishwashing detergent composition may also comprise other enzymes, in particular an amylase, a protease and/or a cellulase.

[0121] The dishwashing detergent composition of the invention may also contain other conventional detergent ingredients, e.g. deflocculant material, filler material, foam depressors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, dehydrating agents, dyes, bactericides, fluorescers, thickeners and perfumes.

[0122] Finally, the variant of the invention may be used in conventional dishwashing detergents, e.g. any of the detergents described in any of the following patent publications:
EP 551670, EP 533239, WO 9303129, EP 507404, US 5141664, GB 2247025, EP 414285, GB 2234980, EP 408278, GB 2228945, GB 2228944, EP 387063, EP 385521, EP 373851, EP 364260, EP 349314, EP 331370, EP 318279, EP 318204, GB 2204319, EP 266904, US 5213706, EP 530870, CA 2006687, EP 481547, EP 337760, WO 93/14183, US 5223179, WO 93/06202, WO 93/05132, WO 92/19707, WO 92/09680, WO 92/08777, WO 92/06161, WO 92/06157, WO 92/06156, WO 91/13959, EP 399752, US 4941988, US 4908148.

[0123] Furthermore, the lipase variants of the invention may be used in softening compositions:

[0124] The lipase variant may be used in fabric softeners, e.g. as described in Surfactant and Consumer Products, Ed. by J. Falbe, 1987, pp 295-296; Tenside Surfactants Detergents, 30 (1993), 6, pp 394-399; JAOCS, Vol. 61 (1984), 2, pp 367-376; EP 517 762; EP 123 400; WO 92/19714; WO 93/19147; US 5,082,578; EP 494 769; EP 544 493; EP 543 562; US 5,235,082; EP 568 297; EP 570 237.

[0125] The invention is further described in the accompanying drawings in which

Fig. 1 is a restriction map of pYESHL,
Fig. 2 a restriction map of the plasmid pAO1,
Fig. 3 a restriction map of the plasmid pAHL, and
Figs. 4 and 5 the construction of genes encoding variant of the invention.

[0126] The invention is further described in the following examples which are not, in any way, intended to limit the

scope of the invention as claimed.

MATERIALS AND METHODS

[0127] *Humicola lanuginosa* DSM 4109 available from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroderweg 1b, D-3300 Braunschweig, Federal Republic of Germany.

[0128] pYESHL is a yeast/*E. coli* shuttle vector that expresses and secretes a low level of the *H. lanuginosa* lipase in yeast. More specifically pYESHL is a derivative of pYES2 (purchased from Invitrogen Corp., UK) in which the GAL1 promoter was excised and the Humicola lanuginosa lipase gene and the TPI (triose phosphate isomerase) promoter from S. cerevisiae (Alber, T. and Kawasaki, G., J.Mol.Appl. Genet 1, 419-434 (1982) were cloned between the SphI and XbaI sites. A restriction map of pYESHL is shown in Fig. 1.

Low calcium filter assay

Procedure

[0129]

1) Provide SC Ura replica plates (useful for selecting strains carrying the expression vector) with a first protein binding filter (Nylon membrane) and a second low protein binding filter (Cellulose acetate) on the top.

2) Spread yeast cells containing a parent lipase gene or a mutated lipase gene on the double filter and incubate for 2 or 3 days at 30°C.

3) Keep the colonies on the top filter by transferring the topfilter to a new plate.

4) Remove the protein binding filter to an empty petri dish.

5) Pour an agarose solution comprising an olive oil emulsion (2% P.V.A.:Olive oil=3:1), Brilliant green (indicator, 0.004%), 100 mM tris buffer pH9 and EGTA (final concentration 5mM) on the bottom filter so as to identify colonies expressing lipase 5 activity in the form of blue-green spots.

6) Identify colonies found in step 5) having a reduced dependency for calcium as compared to the parent lipase.

Dobanol™25-7 filter assay:

[0130] The screening for an improved tolerance towards a detergent component is performed by use of a filter assay corresponding to that described above except for the fact that the solution defined in 5) further comprises 0.02% Dobanol™25-7.

Construction of random mutagenized libraries

a) Using an entire lipase coding gene

[0131] The plasmid pYESHL is treated with 12 M formic acid for 20 min. at room temperature. The resulting lipase encoding gene is amplified from the formic acid treated plasmid using PCR under mutagenic conditions (0.5 mM $MnCl_2$ and 1/5 the normal amount of ATP, see e.g. Leung et al., 1989.

[0132] This treatment is expected to give a broad range of mutations since formic acid gives mainly transversions and PCR generated mutations mainly transitions.

[0133] The resulting PCR fragments are cloned either by double recombination (Muhlrad et al., 1992) in vivo into the shuttle vector or digestion and ligation into the shuttle vector and transformation of *E. coli*.

[0134] Eight randomly picked clones have been sequenced and were found to contain 2-3 mutations in average - both transversion and transitions.

[0135] By use of this method seven libraries have been made containing from 10,000 to 140,000 clones.

b) Performing localized random mutagenesis

[0136] A mutagenic primer (oligonucleotide) is synthesized which corresponds to the part of the DNA sequence to

be mutagenized except for the nucleotide(s) corresponding to amino acid codon(s) to be mutagenized.

[0137] Subsequently, the resulting mutagenic primer is used in a PCR reaction with a suitable opposite primer. The resulting PCR fragment is purified and digested and cloned into the shuttle vector. Alternatively and if necessary, the resulting PCR fragment is used in a second PCR reaction as a primer with a second suitable opposite primer so as to allow digestion and cloning of the mutagenized region into the shuttle vector. The PCR reactions are performed under normal conditions.

[0138] DNA sequencing was performed by using applied Biosystems ABI DNA sequence model 373A according to the protocol in the ABI Dye Terminator Cycle Sequencing kit.

EXAMPLES

EXAMPLE 1

Construction of random lipase variants

[0139] Random mutagenized libraries of the entire *H. lanuginosa* lipase gene and of amino acids (aa) 91-97 and 206-211 thereof were prepared as described in Materials and Methods above.

[0140] The amino acid regions 91-97 and 206-211 were chosen for the first round of localized mutagenesis since these regions have been found to be important for wash performance. Region 91-97 is a part of the lid region of the lipase and region 206-211 constitutes part of the hydrophobic cleft of the lipase.

[0141] One oligonucleotide was synthesized for each of these regions comprising 93% of the wild type nucleotides and 2.33% of each of the other three nucleotides at amino acid codons wanted to be mutagenized. Where possible without changing the amino acid, the third nucleotide (the wobble base) in codons were synthesized with 50%G/50%C to give a larger likelyhood for changes to amino acids with one or two codons. The composition of the mutagenic oligonucleotide of region 91-97 is shown in Table 1.

[0142] By use of this oligonucleotide a calculated mutation frequency of approximately 65-70% is obtained in the library for one amino acid change having been introduced in the parent lipase. The mutation frequency for two or more amino acid changes having been introduced are less than 35 %. This low mutation frequency is chosen to ensure that the observed amino acid changes in positive clones are involved in improving the enzyme and not just "neutral" changes due to a high mutation frequency.

[0143] The mutagenic primer were used in a PCR reaction with a suitable opposite primer. The resulting PCR fragment were purified and in the case of region 206-211 digested and cloned into the shuttle vector. In the case of region 91-97 the resulting PCR fragment was used in a second PCR reaction as a primer with a second suitable opposite primer. This step was necessary to be able to digest and clone the mutagenized region into the shuttle vector.

[0144] Libraries of region 91-97 and of region 206-211 have been prepared containing from 10,000 to 80.000 clones/library. Most colonies were positive (more than 90%) when checked under conditions where the parent lipase is positive, i.e. exhibits lipase activity. The positive reaction was determined in a filter assay with 2.5 mM Ca (instead of 5 mM EGTA).

[0145] 450.000 colonies were screened from the different libraries using the Dobanol™25-7 and low calcium assays described in Materials and Methods above. 25 low calcium positives from the aa 91-97 library (lid-region) and twelve Dobanol™25-7 positives from the whole gene libraries were isolated. Fourteen of the low calcium positives from mutagenesis of aa 91-97 were sequenced.

[0146] The three other mutations (in codon 83, 103, 145), outside the mutagenized region, can be explained by PCR misincoorperation, allthough the mutation of S83T is a transversion which is quite unusual for PCR misincoorperations.

Sequence:

5'	5	C	G
T	5	C	3'
T	7	A	
A	8	G	Bottle 5: 93% A; 2.33% C; 2.33% G and 2.33% T
T	8	T	
T	A/C	T	
T	5	C	
C	7	T	
T	5	C	Bottle 6: 93% C; 2.33% A; 2.33% G and 2.33% T
T	8	T	
T	8	A	
6	C/G	T	
5	6	G	Bottle 7: 93% G; 2.33% A; 2.33% C and 2.33% T
5	6	G	
7	G	A	
8	AA	A	
6	T	C	Bottle 8: 93% T; 2.33% A; 2.33% C and 2.33% G
7			

[0147] **Table 1:** Illustration of the construction of oligonucleotides used for localized random mutagensis of amino acids 91-97 of the *H. lanuginosa* lipase. The numbers presented in the sequence refer to the bottles the composition of which is apppearing to the right of the sequence.

Table 2

Strain number	Variant type						
59	I			G91A	N94K		D96A
60	II	S83T			N94K		D96N
61	II	S83T			N94K		D96N
62	III		E87K				D96V
63	IV		E87K	G91A			D96V
64	II	S83T			N94K		D96N
65	III		E87K				D96V
67	V				N94K	F95L	D96H
69	V				N94K	F95L	D96H
71	III		E87K				D96V
72	II	S83T			N94K		D96N

[0148] **Table 2:** Strain number refers to the originally picked clones cloned into Aspergillus expression vector pAHL.

Variant type refers to identical clones, which probably have arisen during amplification of the random mutagenized library. Variant types I and II are active in 0.01% Dobanol™25-7 while the rest are inactive like wild type.

Table 3

Strain number	Variant type	DNA sequence (Amino acid number above the sequence)										
		82	83	84	85	86	87	88	89	90	91	92
wt		GGC	TCT	CGT	TCC	ATA	GAG	AAC	TGG	ATC	GGG	AAT
59	I										C	
60	II		A								C	
61	II		A								C	
62	III						A				C	
63	IV						A				C	
64	II		A								C	
65	III						A				C	
67	V										C	
52/68	wt											
53	wt											
69	V										C	
71	III						A				C	
72	II		A								C	
73	VI											

Strain number	Variant type	93	94	95	96	97	98	99	100	-103	-145
wt		CTT	AAC	TTC	GAC	TTG	AAA	GAA	ATA	-ATT	-CAT
59	I	G	G		C						
60	II	G	G		A						
61	II	G	G		A						
62	III				T						
63	IV				C					C	C
64	II	G	G		A						
65	III	G			T						
67	V		A	C	A	C					
52/68	wt										
53	wt										
69	V		A	C	A	C					
71	III	G			T						
72	II	G	A		A						
73	VI				A		?				

[0149] **Table 3:** The wildtype seqence is shown at the topline. Only nucleotides differing from wt are written at the variant sequences. The base of codon 91 and 93 were doped with 1:1 of C/T and T/G, respectively. Otherwise the nucleotides at codon 91-97 were doped using 93% wt and 2.33 % of the three other nucleotides.

EXAMPLE 2

[0150] Analogously to the method described in Example 1, the following variants were constructed by random mutagenesis. The actual screening criteria used for selecting some of the variants are also described.

D167G+E210V

5mM EGTA, 0.01% Dobanol™25-7,0.006% LAS

E87K+G91A+L93I+N94K+D96A

5mM EGTA,0.02% Dobanol™25-7
N73D+S85T+E87K+G91A+N94K+D96A
S83T+E87K+W89G+G91A+N94K+D96V
E87K+G91A+D96R+I100V
S83T+E87K+Q249R
E87K+G91A

EXAMPLE 3

Expression of Humicola lanuginosa lipase in *Aspergillus oryzae*

[0151] Cloning of Humicola lanuginosa lipase is described in EP 305 216. It also describes expression and characterization of the lipase in Aspergillus oryzae. The expression plasmid used is named p960.

[0152] The expression plasmid used in this application is identical to p960, except for minor modifications just 3' to the lipase coding region. The modifications were made the following way: p960 was digested with NruI and BamHI restriction enzymes. Between these two sites the BamHI/NheI fragment from plasmid pBR322, in which the NheI fragment was filled in with Klenow polymerase, was cloned, thereby creating plasmid pAOI (figure 2), which contains unique BamHI and NheI sites. Between these unique sites BamHI/XbaI fragments from p960 was cloned to give pAHL (figure 3).

Site-directed in vitro mutagenesis of lipase gene

[0153] The approach used for introducing mutations into the lipase gene is described in Nelson & Long, Analytical Biochemistry, 180, 147-151 (1989). It involves the 3-step generation of a PCR (polymerase chain reaction) fragment containing the desired mutation introduced by using a chemically synthesized DNA-strand as one of the primers in the PCR-reactions. From the PCR generated fragment, a DNA fragment carrying the mutation can be isolated by cleavage with restriction enzymes and re-inserted into the expression plasmid. This method is thoroughly described in Example 5. In figures 4 and 5 the method is further outlined.

Construction of a plasmid expressing the N94K/D96A analogue of *Humicola lanuginosa* lipase

Linearization of plasmid pAHL

[0154] The circular plasmid pAHL is linearized with the restriction enzyme SphI in the following 50 µl reaction mixture: 50 mM NaCl, 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 µg plasmid and 2 units of SphI. The digestion is carried out for 2 hours at 37°C. The reaction mixture is extracted with phenol (equilibrated with Tris-HCl, pH 7.5) and precipitated by adding 2 volumes of ice-cold 96% ethanol. After centrifugation and drying of the pellet, the linearized DNA was dissolved in 50 µl H_2O and the concentration estimated on an agarose gel.

3-step PCR mutagenesis

[0155] As shown in figure 5, 3-step mutagenisation involves the use of four primers:

```
Mutagenisation primer (=A):   5'-TATTTCTTTCAAAGCGAACTTAAGATTC-
                              CCGAT-3'

PCR Helper 1 (=B):            5'-GGTCATCCAGTCACTGAGACCCTCTACCTATTAA-
                              ATCGGC-3'

PCR Helper 2 (=C):            5'-CCATGGCTTTCACGGTGTCT-3'

PCR Handle (=D):              5'-GGTCATCCAGTCACTGAGAC-3'
```

[0156] Helper 1 and helper 2 are complementary to sequences outside the coding region, and can thus be used in combination with any mutagenisation primer in the construction of a variant sequence.

[0157] All 3 steps are carried out in the following buffer containing: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

[0158] In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid is added to a total of 100 µl reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C are carried out.

[0159] The concentration of the PCR product is estimated on an agarose gel. Then, step 2 is carried out. 0.6 pmol step 1 product and 1 fmol linearized plasmid is contained in a total of 100 µl of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C is carried out.

[0160] To the step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D is added (1 µl of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C are carried out. This manipulation comprised step 3 in the mutagenisation procedure.

Isolation of mutated restriction fragment

[0161] The product from step 3 is isolated from an agarose gel and re-dissolved in 20 µl H_2O. Then, it is digested with the restriction enzymes BamHI and BstXI in a total volume of 50 µl with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 10 units of BamHI and 10 units of BstXI. Incubation is at 37°C for 2 hours. The 733 bp BamHI/BstXI fragment is isolated from an agarose gel.

Ligation to expression vector pAHL

[0162] The expression plasmid pAHL is cleaved with BamHI and BstXI under conditions indicated above and the large fragment is isolated from an agarose gel. To this vector, the mutated fragment isolated above is ligated and the ligation mix is used to transform E.coli. The presence and orientation of the fragment is verified by cleavage of a plasmid preparation from a transformant with restriction enzymes. Sequence analysis is carried out on the double-stranded plasmid using the DyeDeoxy™ Terminater Cycle Sequencing Kit (Applied Biosystems) on an ABI DNA sequencer, model 373A. The plasmid is named pAHLG91A/N94K/D96A and is identical to pAHL, except for the substituted codons.

EXAMPLE 4

Construction of plasmids expressing other variants of *Humicola lipase*

[0163] The following variant is constructed using the same method as described in example 3. Plasmid name and primer used for the modification is listed below.

Plasmid name	Primer A sequence
pAHLS83T/N94K/D96A	5′-ATTTCTTTCAAAGCGAACTTAAGATTCCCGA-TCCAGTTCTCTATGGAACGAGTGCCACGGAAAGA-3'
pAHLE87K/D96V	5-TATTTCTTTCAAAACGAAGTTAAGATTCCCGATCC-AGTTCTTTATGGAACGAGA-3'
pAHLE87K/G91A/D96A	5′-TATTTCTTTCAAAGCGAAGTTAAGATTAGCGATC-CAGTTCTTTATGGAACGAGA-3'
pAHLN94K/F95L/D96H	5'-TATTTCTTTCAAGTGCAACTTAAGATTCCCGAT-3'
pAHLF95C/D96N	5'-TATTTCTTTCAAGTTACAGTTAAGATTCCC-3'
pAHLG91S/L93V/F95C	5′-TATTTCTTTCAAGTCACAGTTAACATTAGAGATCC-AGTTCTC-3'
pAHLE87K/G91A/L93I/N94K/D96A	5′-TATTTCTTTCAAAGCGAACTTAATATTAGCGATC-CAGTTCTTTATGGAACGAGA-3'
pAHLD167G	5'-ATATGAAAACACACCGATATCATACCC-3'
pAHLA121V	5'-CCTTAACGTATCAACTACAGACCTCCA-3'
pAHLR205K/E210Q	5'-GCTGTAACCGAATTGGCGCGGCGGGAGCTTAGGG-ACAATATC-3'
pAHLN73D/S85T/E87K/G91A/N94K/D96A	5'-TATTTCTTTCAAAGCGAACTTAAGATTAGCGATC-CAGTTCTTTATAGTACGAGAGCCACGGAA-AGAGAGGACGATCAATTTGTCCGTGTTGTCGAG-3'
pAHLS83T/E87K/W89G/G91A/N94K/D96V	5'-TATTTCTTTCAAAACGAACTTAAGATTAGCGATA-CCGTTCTTTATGGAACGAGTGCCACGGAAAGA-3'
pAHLE87K/G91A/D96R/I100V	5'-GCAAATGTCATTAACTTCTTTCAATCTGAAGTTAA-GATTAGCGATCCAGTTCTTTATGGAACGAGA-3'
pAHLS83T/E87K	5'-CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-AAAGA-3'
pAHLE87K/G91A	5'-GAAGTTAAGATTAGCGATCCAGTTCTTTATGGAA-CGAGA-3'
pAHLS83T/E87K	5'-CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-AAAGA-3'
pAHLQ249R	5'-CGGAATGTTAGGTCTGTTATTGCCGCC-3'

EXAMPLE 5

Construction of plasmids expressing combination analogues of *Humicola lipase*

[0164] The plasmids pAHLD167G/E210V
pAHLA121V/R205K/E210Q
and pAHLS83T/E87K/Q249R
are constructed by performing two successive mutagenisation steps using the appropriate primers.

EXAMPLE 6

Expression of lipase analogues in *Aspergillus*

Transformation of *Aspergillus oryzae* (general procedure)

[0165] 100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) is inoculated with spores of A. oryzae and incubated with shaking for about 24 hours. The mycelium is harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium is suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH = 5.8. The suspension is cooled on ice and 1 ml of buffer containing 120 mg of Novozym 234, batch 1687 is added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) is added and incubation with gentle agitation continued for 1.5 - 2.5 hours at 37°C until a large number of protoplasts is visible in a sample inspected under the microscope.

[0166] The suspension is filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation is performed for 15 min. at 1000 g and the protoplasts are collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$) are added to the protoplast suspension and the mixture is centrifugated for 5 min. at 1000 g. The protoplast pellet is resuspended in 3 ml of STC and repelleted. This is repeated. Finally, the protoplasts are resuspended in 0.2 - 1 ml of STC.

[0167] 100 µl of protoplast suspension is mixed with 5 - 25 µg of p3SR2 (an A. nidulans amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 µl of STC. The mixture is left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH = 7.5 is added and carefully mixed (twice) and finally 0.85 ml of the same solution is added and carefully mixed. The mixture is left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet is resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts are spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4 - 7 days at 37°C spores are picked, suspended in sterile water and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation are stored as a defined transformant.

Expression of lipase analogues in *A. oryzae*

[0168] The plasmids described above are transformed into A. oryzae IFO 4177 by cotransformation with p3SR2 containing the amdS gene from A. nidulans as described in the above example. Protoplasts prepared as described are incubated with a mixture of equal amounts of expression plasmid and p3SR2, approximately 5 µg of each are used. Transformants which could use acetamide as sole nitrogen source are reisolated twice. After growth on YPD for three days, culture supernatants are analyzed using an assay for lipase activity. The best transformant is selected for further studies and grown in a 1 1 shake-flask on 200 ml FG4 medium (3% soy meal, 3% maltodextrin, 1% peptone, pH adjusted to 7.0 with 4 M NaOH) for 4 days at 30°C.

EXAMPLE 7

Purification of lipase variants of the invention

[0169] Assay for lipase activity :
A substrate for lipase was prepared by emulsifying glycerine tributyrat (MERCK) using gum-arabic as emulsifier.

[0170] Lipase activity was assayed at pH 7 using pH stat method. One unit of lipase activity (LU/mg) was defined as the amount needed to liberate one micromole fatty acid per minute.

[0171] Step 1:- Centrifuge the fermentation supernatant, discard the precipitate. Adjust the pH of the supernatant to

7 and add gradually an equal volume of cold 96 % ethanol. Allow the mixture to stand for 30 minutes in an ice bath. Centrifuge and discard the precipitate.

[0172] Step 2:- Ion exchange chromatography. Filter the supernatant and apply on DEAE-fast flow (Pharmacia TM) column equilibrated with 50 mM tris-acetate buffer pH 7. Wash the column with the same buffer till absorption at 280 nm is lower than 0.05 OD. 5 Elute the bound enzymatic activity with linear salt gradient in the same buffer (0 to 0.5 M NaCl) using five column volumes. Pool the fractions containing enzymatic activity.

[0173] Step 3:- Hydrophobic chromatography. Adjust the molarity of the pool containing enzymatic activity to 0.8 M by adding solid Ammonium acetate. Apply the enzyme on TSK gel Butyl- Toyopearl 650 C column (available from Tosoh Corporation Japan) which was pre-equilibrated with 0.8 M ammonium acetate. Wash the unbound material with 0.8 M ammonium acetate and elute the bound material with distilled water.

[0174] Step 4: - Pool containing lipase activity is diluted with water to adjust conductance to 2 mS and pH to 7. Apply the pool on High performance Q Sepharose (Pharmacia) column pre-equilibrated with 50 mM tris -acetate buffer pH 7. Elute the bound enzyme with linear salt gradient.

EXAMPLE 8

The washing performance of lipase variants of the invention

[0175] The washing performance of Humicola lanuginosa lipase variants of the invention was evaluated on the basis of the enzyme dosage in mg of protein per litre according to OD_{280} compared to the wild-type H. lanuginosa lipase.

[0176] Wash trials were carried out in 150 ml beakers placed in a thermostated water bath. The beakers were stirred with triangular magnetic rods.

[0177] The experimental conditions were as follows:

Method	3 cycles with overnight drying between each cycle	
Wash liquor	100 ml per beaker	
Swatches	6 swatches (3.5 x 3.5 cm) per beaker	
Fabric	100% cotton, Test Fabrics style #400	
Stain	Lard coloured with Sudan red (0.75 mg dye/g of lard). 6 µl of lard heated to 70°C was applied to the centre of each swatch. After application of the stain, the swatches were heated in an oven at 75°C for 30 minutes. The swatches were then stored overnight at room temperature prior to the first wash.	
Detergent	LAS (Nansa 1169/P, 30% a.m.)	1.17 g/l
	AEO (Dobanol™ 25-7)	0.15 g/l
	Sodium triphosphate	1.25 g/l
	Sodium sulphate	1.00 g/l
	Sodium carbonate	0.45 g/l
	Sodium silicate	0.15 g/l
pH	10.2	
Lipase conc.	0.075, 0.188, 0.375, 0.75 and 2.5 mg of lipase protein per litre	
Time	20 minutes	
Temperature	30°C	
Rinse	15 minutes in running tap water	
Drying	overnight at room temperature (~20°C, 30-50% RH)	
Evaluation	after the 3rd wash, the reflectance at 460 nm was measured.	

Results

[0178] Dose-response curves were compared for the lipase variants and the native H. lanuginosa lipase. The dose-response curves were calculated by fitting the measured data to the following equation:

$$\Delta R = \Delta R_{max} \frac{C^{0.5}}{K + C^{0.5}} \quad (I)$$

where

ΔR is the effect expressed in reflectance units
C is the enzyme concentration (mg/l)
ΔR_{max} is a constant expressing the maximum effect
K is a constant; K^2 expresses the enzyme concentration at which half of the maximum effect is obtained.

[0179] Based on the characteristic constants ΔR_{max} and K found for each lipase variant as well as the wild-type lipase, improvement factors were calculated. The improvement factor, defined as

$$f_{improve} = C_{WT}/C \quad (II)$$

expresses the amount of lipase variant protein needed to obtain the same effect as that obtained with 0.25 mg/l of the reference wild-type protein (C_{WT}).

[0180] Thus, the procedure for calculating the improvement factor was as follows:

1) The effect of the wild-type protein at 0.25 mg/l ($\Delta R_{wild\text{-}type}$) was calculated by means of equation (I);

2) the concentration of lipase variant resulting in the same effect as the wild-type at 0.25 mg/l was calculated by means of the following equation:

$$C = \left(K_{(analogue)} \frac{\Delta R_{(wild\text{-}type)}}{\Delta R_{max(analogue)} - \Delta R_{(wild\text{-}type)}} \right)^2 \quad (III)$$

3) the improvement factor was calculated by means of equation (II).

[0181] The results are shown in Table 1 below.

Table 1

Variant	Improvement factor
E87K+D96V	1.2
S83T+N94K+D96N	2.3
N94K+D96A	2.7
E87K+G91A+D96A	2.6
N94K+F95L+D96H	3.3
D167G+E210V	5.0
E87K+G91A+L93I+N94K+ D96A	1.3
E87K+G91A+D96R+I100V	5.2
E87K+G91A	5.0
N73D+E87K+G91A+N94I+ D96G	1.3
S83T+E87K+G91A+N92H+ N94K+D96M	3.8
K46R+E56R+G61S	1.9
D102K	0.2
D167G	1

Table 1 (continued)

Variant	Improvement factor
N73D+E87K+G91A+ N94I+D96G	1.3
E210R	2.7
E210K	5.5
E210W	1
N251W+D254W+T267W	0.8
S83T+E87K+G91A+N92H+ N94K+D96M	3.8
E56R+I90F+D96L+E99K	4.8
D57G+N94K+D96L+L97M	1.9

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SEQUENCE LISTING

[0183]

(1) GENERAL INFORMATION:

(i) APPLICANT: Novo Nordisk A/S

(ii) TITLE OF INVENTION: A Method of Preparing a Variant of a Lipolytic Enzyme

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) ZIP: 2880

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 4153.204-WO

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: +45 4444 8888
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(C) TELEX: 37304

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 918 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Humicola lanuginosa

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..873
(C) NAME/KEY: mat_peptide
(D) LOCATION: 67..873

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG AGG AGC TCC CTT GTG CTG TTC TTT GTC TCT GCG TGG ACG GCC TTG   48
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
      -20                 -15                 -10

GCC AGT CCT ATT CGT CGA GAG GTC TCG CAG GAT CTG TTT AAC CAG TTC   96
Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
   -5                  1                   5                  10

AAT CTC TTT GCA CAG TAT TCT GCA GCC GCA TAC TGC GGA AAA AAC AAT  144
Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
               15                  20                  25

GAT GCC CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC  192
Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
           30                  35                  40

GAG GTA GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT  240
Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
       45                  50                  55

GGA GTG GGC GAT GTC ACC GGC TTC CTT GCT CTC GAC AAC ACG AAC AAA  288
Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
   60                  65                  70

TTG ATC GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAG AAC TGG ATC  336
Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
75                  80                  85                  90

GGG AAT CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC  384
Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
               95                  100                 105

TGC AGG GGA CAT GAC GGC TTC ACT TCG TCC TGG AGG TCT GTA GCC GAT  432
Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
           110                 115                 120
ACG TTA AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT  480
Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
       125                 130                 135

CGC GTG GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT  528
Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
   140                 145                 150

GCC GGA GCA GAC CTG CGT GGA AAT GGG TAT GAT ATC GAC GTG TTT TCA  576
Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155                 160                 165                 170

TAT GGC GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC  624
Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
               175                 180                 185

GTA CAG ACC GGC GGA ACA CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT  672
Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
           190                 195                 200

GTC CCT AGA CTC CCG CCG CGC GAA TTC GGT TAC AGC CAT TCT AGC CCA  720
Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
       205                 210                 215

GAG TAC TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAT  768
Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
   220                 225                 230

ATC GTG AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT  816
Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

AAC ATT CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGC  864
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Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
                255                 260                 265

ACA TGT CTT TAGTGGCCGG CGCGGCTGGG TCCGACTCTA GCGAGCTCGA GATCT    918
Thr Cys Leu
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 291 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
-20                 -15                 -10

Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
-5                  1                   5                   10

Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
                15                  20                  25

Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
            30                  35                  40

Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
        45                  50                  55

Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
    60                  65                  70

Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
75                  80                  85                  90

Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
                95                  100                 105

Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
            110                 115                 120

Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
        125                 130                 135

Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
    140                 145                 150

Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155                 160                 165                 170

Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
                175                 180                 185

Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
            190                 195                 200

Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
        205                 210                 215

Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
    220                 225                 230

Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
                255                 260                 265

Thr Cys Leu
```

Claims

1. A method of preparing a variant of a parent lipolytic enzyme, which method comprises

   a) subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis,

b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
c) screening for host cells expressing a mutated lipolytic enzyme which has a decreased dependance to calcium.

2. The method according to claim 1, wherein step (c) further comprises screening for an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme.

3. The method according to claim 1 or 2, in which the random mutagenesis is performed by use of a physical or a chemical mutagenizing agent, by use of an oligonucleotide or by use of PCR generated mutagenesis.

4. The method according to claim 3, in which the mutagenizing agent is selected from formic acid, UV irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, and nucleotide analogues.

5. The method according to claim 1, in which the expression of mutated DNA sequence is performed by transforming a suitable host cell with the mutated DNA sequence, the mutated DNA sequence optionally further comprising a DNA sequence encoding functions permitting expression of the mutated DNA sequence, and culturing the host cell obtained in step (b) under suitable conditions for expressing the mutated DNA sequence.

6. The method according to claim 1, in which the host cell used for expressing the mutated DNA sequence is a microbial cell.

7. The method according to claim 6, in which the host cell is a cell of a fungal or a bacterial strain.

8. The method according to claim 7, in which the host cell is a cell of the genus *Aspergillus,* such as *A. niger, A. oryzae* and *A. nidulans,* or a cell of the genus *Saccharomyces,* e.g. *S. cereviciae.*

9. The method according to claim 7, in which the host cell is a cell of a gram-positive bacterial strain, e.g. of the genus *Bacillus,* such as *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus,* or a cell of a gram-negative bacterial strain, such as *E. coli.*

10. The method according to claim 2, in which the mutated lipolytic enzyme has an improved tolerance towards a non-ionic, anionic, kationic, zwitterionic or amphoteric surfactant.

11. The method according to claim 10, in which the non-ionic surfactant is an alcohol ethoxylate and/or the anionic surfactant is LAS or an alkyl sulphate.

12. The method according to claim 1, wherein host cells screened in step (c) are subjected to a second mutagenesis treatment, to rescreening, to reisolation and/or to recloning.

13. The method according to any of claims 1-12, in which the random mutagenesis is localized to a part of the DNA sequence encoding the parent lipolytic enzyme.

14. The method according to any of claims 1-13, in which the parent lipolytic enzyme is a lipase, an esterase, a cutinase or a phospholipase.

15. The method according to claim 13 or 14, in which the parent lipolytic enzyme is a lipase and the localized random mutagenesis is performed on a part of the DNA sequence encoding a lipid contact zone or a part thereof of the parent lipase.

16. The method according to claim 15, in which the localized random mutagenesis is performed on a part of the DNA sequence encoding a lid region and/or a hydrophobic cleft of the parent lipase or a part of said lid region and/or hydrophibic binding cleft.

17. The method according to any of claims 1-16, wherein the parent lipolytic enzyme is derivable from a microorganism.

18. The method according to claim 17, wherein the parent lipolytic enzyme is derivable from a fungus.

19. The method according to claim 18, wherein the DNA sequence encoding the parent lipolytic enzyme is derivable from a strain of *Humicola sp., Rhizomucor sp., Rhizopus sp.* or *Candida sp.*

20. The method according to claim 19, wherein the parent lipolytic enzyme is a lipase and the DNA sequence encoding the parent lipase is derivable from a strain of *H. lanuginosa*, e.g. the *H. lanuginosa* strain DSM 4109, a strain of *Rh. mucor,* or a strain of *C. antarctica.*

21. The method according to claim 20, in which the DNA sequence subjected to random mutagenesis encodes at least one of the regions defined by the amino acid residues 21-27, 56-64, 81-99, 108-116, 145-147, 174, 202-213, 226-227, 246-259 or 263-269 of the *H. lanuginosa* lipase obtainable from DSM 4109.

22. The method according to claim 21, in which the localized random mutagenesis is performed in at least two of the said regions.

23. The method according to claim 17, wherein the parent lipolytic enzyme is derivable from a bacterium.

24. The method according to claim 23, wherein the DNA sequence encoding the parent lipolytic enzyme is derivable from a strain of *Pseudomonas spp.*, such as *P. cepacia, P. alcaligenes, P. pseudoalcaligens or P. fragi* or from a strain of *Bacillus.*

25. A variant of the *H. lanuginosa* lipase obtainable from DSM 4109, which comprises at least one of the following mutations:

S83T, G91A, I100V, D167G,

and which optionally further comprises the addition of one or more amino acid residues to either or both the N- and C-terminal end of the lipase, substitution of one or more amino acid residues at one or more different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the lipase or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence, provided that the variant retains lipase activity.

26. A variant of the *H. lanuginosa* lipase obtainable from DSM 4109, which comprises at least one of the following sets of mutations:

N94K+D96A
S83T+N94K+D96N
E87K+D96V
E87K+G91A+D96A
N94K+F95L+D96H
F95C+D96N
E87K+G91A+D96R+I100V
E87K+G91A
S83T+E87K+Q249R
S83T+E87K+W89G+G91A+N94K+D96V
N73D+S85T+E87K+G91A+N94K+D94A
E87K+G91A+L93I+N94K+D96A
D167G+E210V
N73D+E87K+G91A+N94I+D96G
S83T+E87K+G91A+N92H+N94K+D96M
E56R+D57L+V60M+D62N+S83T+D96P+D102E
D57G+N94K+D96L+L97M
E87K+G91A+D96R+I100V+E129K+K237M+I252L+P256T+G263A+L264Q
E56R+D57G+S58F+D62C+T64R+E87G+G91A+F95L+D96P+K98I+K237M
D167G
N73D+E87K+G91A+N94I+D96G
S83T+E87K+G91A+N92H+N94K+D96M
G91A+N94K+D96A,

and which optionally further comprises the addition of one or more amino acid residues to either or both the N- and C-terminal end of the lipase, substitution of one or more amino acid residues at one or more different sites

in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the lipase or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence, provided that the variant retains lipase activity.

27. A DNA construct encoding a *H. lanuginosa* lipase variant according to claim 25 or 26.

28. A vector harbouring a DNA construct according to claim 27.

29. The vector according to claim 28, which is a plasmid or a bacteriophage.

30. The vector according to claim 28 or 29, which is an expression vector further comprising DNA sequences permitting expression of the variant of the parent lipolytic enzyme.

31. A host cell harbouring a DNA construct according to claim 27 or a vector according to any of claims 28-30.

32. The cell according to claim 31, which is a microbial cell.

33. The cell according to claim 32, which is a cell of a fungal or a bacterial strain.

34. The cell according to claim 33, which is a cell of the genus *Aspergillus*, such as *A. niger, A. oryzae* or *A. nidulans*, or a cell of the genus *Saccharomyces*, e.g. *S. cereviciae*.

35. The cell according to claim 33, which is a cell of a grampositive bacterial strain, e.g. of the genus *Bacilllus*, such as *Bacillus subtilis*, *Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a cell of a gram-negative bacterial strain, such *as E. colt.*

36. A method of producing a variant of a parent lipolytic enzyme which has a decreased dependance to calcium and optionally an improved tolerance towards a detergent or a detergent component compared to the parent lipolytic enzyme, which method comprises preparing a variant lipolytic enzyme in accordance with the method of any of claims 1-24 and recovering the lipolytic enzyme variant from the host cell screened in step (c).

37. A method of producing a variant of a parent lipolytic enzyme which has a decreased dependance to calcium and optionally an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme, which method comprises culturing a host cell according to any of claims 31-35 under suitable conditions to express the variant, and recovering the expressed variant from the culture.

38. A detergent additive comprising a lipase variant according to claim 25 or 26, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

39. A detergent additive according to claim 38 which contains 0.02-200 mg of enzyme protein/g of the additive.

40. A detergent additive according to claim 38 or 39 which additionally comprises another enzyme such as a protease, amylase, peroxidase, cutinase, lipase and/or cellulase.

41. A detergent composition comprising a lipase variant according to claim 25 or 26.

42. A detergent composition according to claim 41 which additionally comprises another enzyme such as a protease, amylase, peroxidase, cutinase, lipase and/or cellulase.

Patentansprüche

1. Verfahren zur Herstellung einer Variante eines lipolytischen Ausgangsenzyms, wobei das Verfahren umfasst

   a) Behandeln einer DNA-Sequenz kodierend für das lipolytische Ausgangsenzym mit Zufallsmutagenese,
   b) Exprimieren der mutierten DNA-Sequenz erhalten in Schritt a) in einer Wirtszelle, und

c) Screening nach Wirtszellen, die ein mutiertes lipolytisches Enzym exprimieren, das eine verminderte Kalziumabhängigkeit hat.

2. Verfahren nach Anspruch 1, wobei Schritt c) zusätzlich das Screening nach verbesserter Toleranz gegenüber einem Detergens oder einer Detergenskomponente, im Vergleich zum lipolytischen Ausgangsenzym, umfasst.

3. Verfahren nach Anspruch 1 oder 2, wobei die Zufallsmutagenese durchgeführt wird durch Verwendung eines physikalischen oder chemischen mutagenisierenden Agens, durch Verwendung eines Oligonukleotids oder durch Verwendung von PCR-vermittelter Mutagenese.

4. Verfahren nach Anspruch 3, wobei das mutagenesierende Agens ausgewählt ist aus Ameisensäure, UV-Bestrahlung, Hydroxylamin, N-Methyl-N'-nitro-N-nitrosoguanidin (MNNG), O-Methylhydroxylamin, Salpetriger Säure, Ethylmethansulfonat (EMS), Natriumbisulfit, und Nukleotidanaloga.

5. Verfahren nach Anspruch 1, wobei die Expression der mutierten DNA-Sequenz durchgeführt wird durch Transformation einer geeigneten Wirtszelle mit der mutierten DNA-Sequenz, wobei die mutierte DNA-Sequenz wahlweise weiterhin eine DNA-Sequenz umfasst, die für Funktionen kodiert, die die Expression der mutierten DNA-Sequenz erlauben, und Kultivierung der in Schritt b) erhaltenen Wirtszelle unter für die Expression der mutierten DNA-Sequenz geeigneten Bedingungen.

6. Verfahren nach Anspruch 1, wobei die für die Expression der mutierten DNA-Sequenz verwendete Wirtszelle eine mikrobielle Zelle ist.

7. Verfahren nach Anspruch 6, wobei die Wirtszelle eine Zelle eines Pilz- oder Bakterienstammes ist.

8. Verfahren nach Anspruch 7, wobei die Wirtszelle eine Zelle des Genus *Aspergillus,* wie *A. niger, A. oryzae* und *A. nidulans,* oder eine Zelle des Genus *Saccharomyces,* z.B. *S. cereviciae,* ist.

9. Verfahren nach Anspruch 7, wobei die Wirtszelle eine Zelle eines Gram-positiven Bakterienstamms ist, z.B. des Genus *Bacillus,* wie *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis* oder *Streptomyces lividans* oder *Streptomyces murinus,* oder eine Zelle eines Gram-negativen Bakterienstammes, wie *E. coli,* ist.

10. Verfahren nach Anspruch 2, wobei das mutierte lipolytische Enzym eine verbesserte Toleranz gegenüber einem nicht-ionischen, anionischen, kationischen, zwitterionischen oder amphotherischen Tensid hat.

11. Verfahren nach Anspruch 10, wobei das nicht-ionische Tensid ein Alkoholethoxylat und/oder das anionische Tensid LAS oder ein Alkylsulfat ist.

12. Verfahren nach Anspruch 1, wobei die in Schritt c) gescreenten Wirtszellen einer zweiten Mutagenesebehandlung, einem Re-Screening, einer Re-Isolation und/oder einer Re-Klonierung unterzogen werden.

13. Verfahren nach einem der Ansprüche 1-12, wobei die Zufallsmutagenese lokalisiert ist auf einen Teil der DNA-Sequenz, die das lipolytische Ausgangsenzym kodiert.

14. Verfahren nach einem der Ansprüche 1-13, wobei das lipolytische Ausgangsenzym eine Lipase, eine Esterase, eine Cutinase oder eine Phospholipase ist.

15. Verfahren nach Anspruch 13 oder 14, wobei das lipolytische Ausgangsenzym eine Lipase ist und die lokalisierte Zufallsmutagenese an einem Teil der DNA-Sequenz durchgeführt wird, der eine Lipidkontaktzone oder einen Teil davon in der Ausgangslipase kodiert.

16. Verfahren nach Anspruch 15, wobei die lokalisierte Zufallsmutagenese durchgeführt wird an einem Teil der DNA-Sequenz, der für eine Deckel-Region und/oder eine hydrophobe Spalte der Ausgangslipase kodiert, oder einen Teil dieser Deckel-Region und/oder hydrophoben Bindungsspalte.

17. Verfahren nach einem der Ansprüche 1-16, wobei das lipolytische Ausgangsenzym von einem Mikroorganismus

erhältlich ist.

18. Verfahren nach Anspruch 17, wobei das lipolytische Ausgangsenzym von einem Pilz erhältlich ist.

19. Verfahren nach Anspruch 18, wobei die DNA-Sequenz, die das lipolytische Ausgangsenzym kodiert, erhältlich ist von einem Stamm von *Humicola sp., Rhizomucor sp., Rhizopus sp.* oder *Candida sp.*

20. Verfahren nach Anspruch 19, wobei das lipolytische Ausgangsenzym eine Lipase ist und die DNA-Sequenz, die für die Ausgangslipase kodiert, erhältlich ist von einem Stamm von *H. lanuginosa*, *z.B.* den *H. lanuginosa* Stamm DSM 4109, einem Stamm von *Rh. mucor*, oder einem Stamm von *C. antarctica.*

21. Verfahren nach Anspruch 20, wobei die der Zufallsmutagenese unterzogene DNA-Sequenz kodiert für zumindest eine der Regionen definiert durch die Aminosäurereste 21-27, 56-64, 81-99, 108-116, 145-147, 174, 202-213, 226-227, 246-259, oder 263-269 der *H. lanuginosa* Lipase erhältlich von DSM 4109.

22. Verfahren nach Anspruch 21, wobei die lokalisierte Zufallsmutagenese in zumindest zwei der genannten Regionen durchgeführt wird.

23. Verfahren nach Anspruch 17, wobei das lipolytische Ausgangsenzym von einem Bakterium erhältlich ist.

24. Verfahren nach Anspruch 23, wobei die DNA-Sequenz, die das lipolytische Ausgangsenzym kodiert, erhältlich ist von einem Stamm von *Pseudomonas spp.*, wie *P. cepacia, P. alcaligenes, P. pseudolacaligens* oder *P. fragi* oder von einem Stamm von *Bacillus.*

25. Variante der *H. lanuginosa* Lipase, erhältlich von DSM 4109, die zumindest eine der folgenden Mutationen umfasst: S83T, G91A, I100V, D167G, und die wahlweise weiterhin umfasst die Addition von einer oder mehreren Aminosäureresten an entweder das N- oder C-terminale Ende der Lipase, oder an beide, die Substitution von einer oder mehreren Aminosäureresten an einer oder mehreren verschiedenen Stellen in der Aminosäuresequenz, Deletion von einer oder mehreren Aminosäureresten an einem oder an beiden Enden der Lipase oder an einer oder mehreren Stellen in der Aminosäuresequenz oder Insertion von einer oder mehreren Aminosäureresten an einer oder mehreren Stellen in der Aminosäuresequenz, vorausgesetzt, dass die Variante Lipaseaktivität behält.

26. Variante der *H. lanuginosa* Lipase erhältlich von DSM 4109 die zumindest eine der folgenden Gruppen von Mutationen umfasst:
N94K+D96A
S83T+N94K+D96N
E87K+D96V
E87K+G91A+D96A
N94K+F95L+D96H
F95C+D96N
E87K+G91A+D96R+I100V
E87K+G91A
S83T+E87K+Q249R
S83T+E87K+W89G+G91A+N94K+D96V
N73D+S85T+E87K+G91A+N94K+D94A
E87K+G91A+L93I+N94K+D96A
D167G+E210V
N73D+E87K+G91A+N94I+D96G
S83T+E87K+G91A+N92H+N94K+D96M
E56R+D57L+V60M+D62N+S83T+D96P+D102E
D57G+N94K+D96L+L97M
E87K+G91A+D96R+I100V+E129K+K237M+I252L+P256T+G263A+L264Q
E56R+D57G+S58F+D62C+T64R+E87G+G91A+F95L+D96P+K98I+K237M
D167G
N73D+E87K+G91A+N94I+D96G
S83T+E87K+G91A+N92H+N94K+D96M
G91A+N94K+D96A,
und die wahlweise zusätzlich die Addition von einem oder mehreren Aminosäureresten an entweder das N- oder

das C-terminale Ende der Lipase, oder an beide, umfasst, Substitution von einer oder mehreren Aminosäureresten an einer oder mehreren verschiedenen Stellen in der Aminosäuresequenz, Deletion von einer oder mehreren Aminosäureresten an einem oder beiden Enden der Lipase oder an einer oder mehreren Stellen in der Aminosäuresequenz oder Insertion von einem oder mehreren Aminosäureresten an einer oder mehreren Stellen in der Aminosäuresequenz, vorausgesetzt, dass die Variante Lipaseaktivität behält.

27. DNA-Konstrukt kodierend für eine *H. lanuginosa* Lipasevariante nach Anspruch 25 oder 26.

28. Vektor beinhaltend ein DNA-Konstrukt nach Anspruch 27.

29. Vektor nach Anspruch 28, der ein Plasmid oder ein Bakteriophage ist.

30. Vektor nach Anspruch 28 oder 29, der ein Expressionsvektor ist, der weiterhin DNA-Sequenzen, die die Expression der Variante des lipolytischen Ausgangsenzyms gestatten, umfasst.

31. Wirtszelle beinhaltend ein DNA-Konstrukt nach Anspruch 27 oder ein Vektor nach irgendeinem der Ansprüche 28 bis 30.

32. Zelle nach Anspruch 31, die eine mikrobielle Zelle ist.

33. Zelle nach Anspruch 32, die eine Zelle eines Pilz- oder Bakterienstammes ist.

34. Zelle nach Anspruch 33, die eine Zelle des Genus *Aspergillus* ist, wie *A. niger, A. oryzae* oder *A. nidulans,* oder eine Zelle des Genus *Saccharomyces,* z.B. *S. cereviciae.*

35. Zelle nach Anspruch 33, die eine Zelle eines Gram-positiven Bakterienstammes ist, z.B. des Genus *Bacilllus*, wie *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis* oder *Streptomyces lividans* oder *Streptomyces murinus,* oder eine Zelle eines Gram-negativen Bakterienstammes, wie *E. coli,* ist

36. Verfahren zur Herstellung einer Variante eines lipolytischen Ausgangsenzyms, die eine verminderte Kalziumabhängigkeit und wahlweise eine verbesserte Toleranz gegenüber einem Detergens oder einer Detergenskomponente im Vergleich zum lipolytischen Ausgangsenzym hat, wobei das Verfahren die Herstellung einer Variante eines lipolytischen Enzyms gemäß dem Verfahren eines der Ansprüche 1 bis 24 sowie die Gewinnung der Variante des lipolytischen Enzyms aus der Wirtszelle, die in Schritt c) gescreent wurde, umfasst.

37. Verfahren zur Herstellung einer Variante eines lipolytischen Ausgangsenzyms, die eine verringerte Kalziumabhängigkeit und wahlweise eine verbesserte Toleranz gegenüber einem Detergens oder einer Detergenskomponente im Vergleich zum lipolytischen Ausgangsenzym hat, wobei das Verfahren das Kultivieren einer Wirtszelle gemäß einem der Ansprüche 31 bis 35 unter für die Expression der Variante geeigneten Bedingungen, sowie die Gewinnung der exprimierten Variante aus der Kultur, umfasst.

38. Detergensadditiv umfassend eine Lipasevariante nach Anspruch 25 oder 26, wahlweise in der Form eines nichtstaubenden Granulats, einer stabilisierten Flüssigkeit oder eines geschützten Enzyms.

39. Detergensadditiv nach Anspruch 38, das 0,02-200 mg des Enzymproteins/g des Additivs enthält.

40. Detergensadditiv nach Anspruch 38 oder 39, das zusätzlich ein weiteres Enzym wie eine Protease, Amylase, Peroxidase, Cutinase, Lipase und/oder Cellulase umfasst.

41. Detergenszusammensetzung umfassend eine Lipasevariante nach Anspruch 25 oder 26.

42. Detergenszusammensetzung nach Anspruch 41, die zusätzlich ein weiteres Enzym wie eine Protease, Amylase, Peroxidase, Cutinase, Lipase und/oder Cellulase umfasst.

Revendications

1. Procédé de préparation d'un variant d'une enzyme lipolytique mère, lequel procédé comprend

   a) l'exposition d'une séquence d'ADN codant l'enzyme lipolytique mère à une mutagenèse aléatoire,
   b) l'expression de la séquence d'ADN mutée obtenue dans l'étape (a) dans une cellule hôte, et
   c) la sélection de cellules hôtes exprimant une enzyme lipolytique mutée qui a une dépendance à l'égard du calcium diminuée.

2. Procédé selon la revendication 1 où l'étape (c) comprend en outre une sélection concernant une tolérance améliorée à l'égard d'un détergent ou d'un composant de détergent par rapport à l'enzyme lipolytique mère.

3. Procédé selon la revendication 1 ou 2 où la mutagenèse aléatoire est réalisée au moyen d'un agent mutagène physique ou chimique, au moyen d'un oligonucléotide ou au moyen d'une mutagenèse générée par PCR.

4. Procédé selon la revendication 3 où l'agent mutagène est choisi parmi l'acide formique, une irradiation UV, l'hydroxylamine, la N-méthyl-N'-nitro-N-nitrosoguanidine (MNNG), la O-méthylhydroxylamine, l'acide nitreux, le méthanesulfonate d'éthyle (EMS), le bisulfite de sodium et des analogues de nucléotides.

5. Procédé selon la revendication 1 où l'expression de la séquence d'ADN mutée est réalisée par transformation d'une cellule hôte appropriée avec la séquence d'ADN mutée, la séquence d'ADN mutée comprenant éventuellement en outre une séquence d'ADN codant des fonctions permettant l'expression de la séquence d'ADN mutée, et culture de la cellule hôte obtenue dans l'étape (b) dans des conditions appropriées pour l'expression de la séquence d'ADN mutée.

6. Procédé selon la revendication 1 où la cellule hôte utilisée pour l'expression de la séquence d'ADN mutée est une cellule microbienne.

7. Procédé selon la revendication 6 où la cellule hôte est une cellule d'une souche fongique ou bactérienne.

8. Procédé selon la revendication 7 où la cellule hôte est une cellule du genre *Aspergillus,* comme *A. niger, A. oryzae* et *A. nidulans,* ou une cellule du genre *Saccharomyces,* par exemple *S. cerevisiae.*

9. Procédé selon la revendication 7 où la cellule hôte est une cellule d'une souche bactérienne gram-positive, par exemple du genre *Bacillus,* comme *Bacillus subtilis, Bacillus licheniformis, Bacillus lentes, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis* ou *Streptomyces lividans* ou *Streptomyces murinus,* ou une cellule d'une souche bactérienne gram-négative, comme *E. coli.*

10. Procédé selon la revendication 2 où l'enzyme lipolytique mutée a une tolérance améliorée à l'égard d'un tensioactif non ionique, anionique, cationique, zwitterionique ou amphotère.

11. Procédé selon la revendication 10 où le tensioactif non ionique est un éthoxylat d'alcool et/ou le tensioactif anionique est un LAS ou un sulfate d'alkyle.

12. Procédé selon la revendication 1 où les cellules hôtes sélectionnées dans l'étape (c) sont soumises à un second traitement de mutagenèse, à une nouvelle sélection, à un nouvel isolement et/ou à un nouveau clonage.

13. Procédé selon l'une quelconque des revendications 1-12 où la mutagenèse aléatoire est localisée à une partie de la séquence d'ADN codant l'enzyme lipolytique mère.

14. Procédé selon l'une quelconque des revendications 1-13 où l'enzyme lipolytique mère est une lipase, une estérase, une cutinase ou une phospholipase.

15. Procédé selon la revendication 13 ou 14 où l'enzyme lipolytique mère est une lipase et la mutagenèse aléatoire localisée est réalisée sur une partie de la séquence d'ADN codant une zone de contact de lipides de la lipase mère, ou une partie de celle-ci.

16. Procédé selon la revendication 15 où la mutagenèse aléatoire localisée est réalisée sur une partie de la séquence d'ADN codant une région couvercle et/ou une fente hydrophobe de la lipase mère ou une partie de ladite région couvercle et/ou de la fente de liaison hydrophobe.

17. Procédé selon l'une quelconque des revendications 1-16 où l'enzyme lipolytique mère peut être obtenue à partir d'un microorganisme.

18. Procédé selon la revendication 17 où l'enzyme lipolytique mère peut être obtenue à partir d'un champignon.

19. Procédé selon la revendication 18 où la séquence d'ADN codant l'enzyme lipolytique mère peut être obtenue à partir d'une souche de *Humicola sp., Rhizomucor sp., Rhizopus sp.* ou *Capdida sp.*

20. Procédé selon la revendication 19 où l'enzyme lipolytique mère est une lipase et la séquence d'ADN codant la lipase mère peut être obtenue à partir d'une souche de *H. lanuginosa,* par exemple la souche DSM 4109 de *H. lanuginosa,* une souche de *Rh. Mucor* ou une souche de *C. antarctica.*

21. Procédé selon la revendication 20 où la séquence d'ADN soumise à une mutagenèse aléatoire code au moins l'une des régions définies par les résidus d'acides aminés 21-27, 56-64, 81-99, 108-116, 145-147, 174, 202-213, 226-227, 246-259 ou 263-269 de la lipase de *H. lanuginosa* qui peut être obtenue à partir de DSM 4109.

22. Procédé selon la revendication 21 où la mutagenèse aléatoire localisée est réalisée dans au moins deux desdites régions.

23. Procédé selon la revendication 17 où l'enzyme lipolytique mère peut être obtenue à partir d'une bactérie.

24. Procédé selon la revendication 23 où la séquence d'ADN codant l'enzyme lipolytique mère peut être obtenue à partir d'une souche de *Pseudomonas spp.*, comme *P. cepacia, P. alcaligenes, P. pseudoalcaligenes* ou *P. fragi* ou à partir d'une souche de *Bacillus.*

25. Variant de la lipase de *H. lanuginosa* qui peut être obtenue à partir de DSM 4109, qui comprend au moins l'une des mutations suivantes :

S83T, G91A, I100V, D167G,

et qui comprend éventuellement en outre l'addition d'un ou plusieurs résidus d'acides aminés à l'une ou l'autre des extrémités N-terminale et C-terminale de la lipase ou aux deux extrémités, la substitution d'un ou plusieurs résidus d'acides aminés à un ou plusieurs sites différents dans la séquence d'acides aminés, la délétion d'un ou plusieurs résidus d'acides aminés à l'une ou l'autre des extrémités ou aux deux extrémités de la lipase ou à un ou plusieurs sites dans la séquence d'acides aminés, ou l'insertion d'un ou plusieurs résidus d'acides aminés à un ou plusieurs sites dans la séquence d'acides aminés, à condition que le variant conserve une activité lipase.

26. Variant de la lipase de *H. lanuginosa* qui peut être obtenue à partir de DSM 4109, qui comprend au moins l'un des ensembles de mutations suivants :

N94K+D96A
S83T+N94K+D96N
E87K+D96V
E87K+G91A+D96A
N94K+F95L+D96H
F95C+D96N
E87K+G91A+D96R+I100V
E87K+G91A
S83T+E87K+Q249R
S83T+E87K+W89G+G91A+N94K+D96V
N73D+S85T+E87K+G91A+N94K+D94A
E87K+G91A+L931+N94K+D96A
D167G+E210V
N73D+E87K+G91A+N941+D96G
S83T+E87K+G91A+N92H+N94K+D96M
E56R+D57L+V60M+D62N+S83T+D96P+D192E
D57G+N94K+D96L+L97M

E87K+G91A+D96R+I100V+E129K+K237M+I252L+P256T+G263A+L264Q
E56R+D57G+S58F+D62C+T64R+E87G+G91A+F95L+D96P+K98I+K237M
N73D+E87K+G91A+N94I+D96G
S83T+E87K+G91A+N92H+N94K+D96M
G91A+N94K+D96A,
et qui comprend éventuellement en outre l'addition d'un ou plusieurs résidus d'acides aminés à l'une ou l'autre des extrémités N-terminale et C-terminale de la lipase, ,ou aux deux extrémités, la substitution d'un ou plusieurs résidus d'acides aminés à un ou plusieurs sites différents dans la séquence d'acides aminés, la délétion d'un ou plusieurs résidus d'acides aminés à l'une ou l'autre des extrémités ou aux deux extrémités de la lipase ou à un ou plusieurs sites dans la séquence d'acides aminés, ou l'insertion d'un ou plusieurs résidus d'acides aminés à un ou plusieurs sites dans la séquence d'acides aminés, à condition que le variant conserve une activité lipase.

27. Construction d'ADN codant un variant de lipase de *H. lanuginosa* selon la revendication 25 ou 26.

28. Vecteur contenant une construction d'ADN selon la revendication 27.

29. Vecteur selon la revendication 28 qui est un plasmide ou un bactériophage.

30. Vecteur selon la revendication 28 ou 29 qui est un vecteur d'expression comprenant en outre des séquences d'ADN permettant l'expression du variant de l'enzyme lipolytique mère.

31. Cellule hôte contenant une construction d'ADN selon la revendication 27 ou un vecteur selon l'une quelconque des revendications 28-30.

32. Cellule selon la revendication 31 qui est une cellule microbienne.

33. Cellule selon la revendication 32 qui est une cellule d'une souche fongique ou bactérienne.

34. Cellule selon la revendication 33 qui est une cellule du genre *Aspergillus,* comme *A. niger, A. oryzae* ou *A. nidulans*, ou une cellule du genre *Saccharomyces,* par exemple *S. cerevisiae.*

35. Cellule selon la revendication 33 qui est une cellule d'une souche bactérienne gram-positive, par exemple du genre *Bacillus,* comme *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus thuringiensis* ou *Streptomyces lividans* ou *Streptomyces murinus,* ou une cellule d'une souche bactérienne gram-négative, comme *E. coli.*

36. Procédé de production d'un variant d'une enzyme lipolytique mère qui a une dépendance diminuée à l'égard du calcium et éventuellement une tolérance améliorée à l'égard d'un détergent ou d'un composant de détergent par rapport à l'enzyme lipolytique mère, lequel procédé comprend la préparation d'une enzyme lipolytique constituant un variant par le procédé selon l'une quelconque des revendications 1-24 et la récupération du variant d'enzyme lipolytique à partir de la cellule hôte sélectionnée dans l'étape (c).

37. Procédé de production d'un variant d'une enzyme lipolytique mère qui a une dépendance diminuée à l'égard du calcium et éventuellement une tolérance améliorée à l'égard d'un détergent ou d'un composant de détergent par rapport à l'enzyme lipolytique mère, lequel procédé comprend la culture d'une cellule hôte selon l'une quelconque des revendications 31-35 dans des conditions appropriées pour exprimer le variant, et la récupération du variant exprimé à partir de la culture.

38. Additif de détergent comprenant un variant de lipase selon la revendication 25 ou 26, éventuellement sous forme de granulés ne produisant pas de poussière, d'un liquide stabilisé ou d'une enzyme protégée.

39. Additif de détergent selon la revendication 38 qui contient 0,02-200 mg de protéine enzyme/g d'additif.

40. Additif de détergent selon la revendication 38 ou 39 qui comprend en outre une autre enzyme comme une protéase, une amylase, une peroxydase, une cutinase, une lipase et/ou une cellulase.

41. Composition de détergent comprenant un variant de lipase selon la revendication 25 ou 26.

42. Composition de détergent selon la revendication 41 qui comprend en outre une autre enzyme comme une protéase, une amylase, une peroxydase, une cutinase, une lipase et/ou une cellulase.

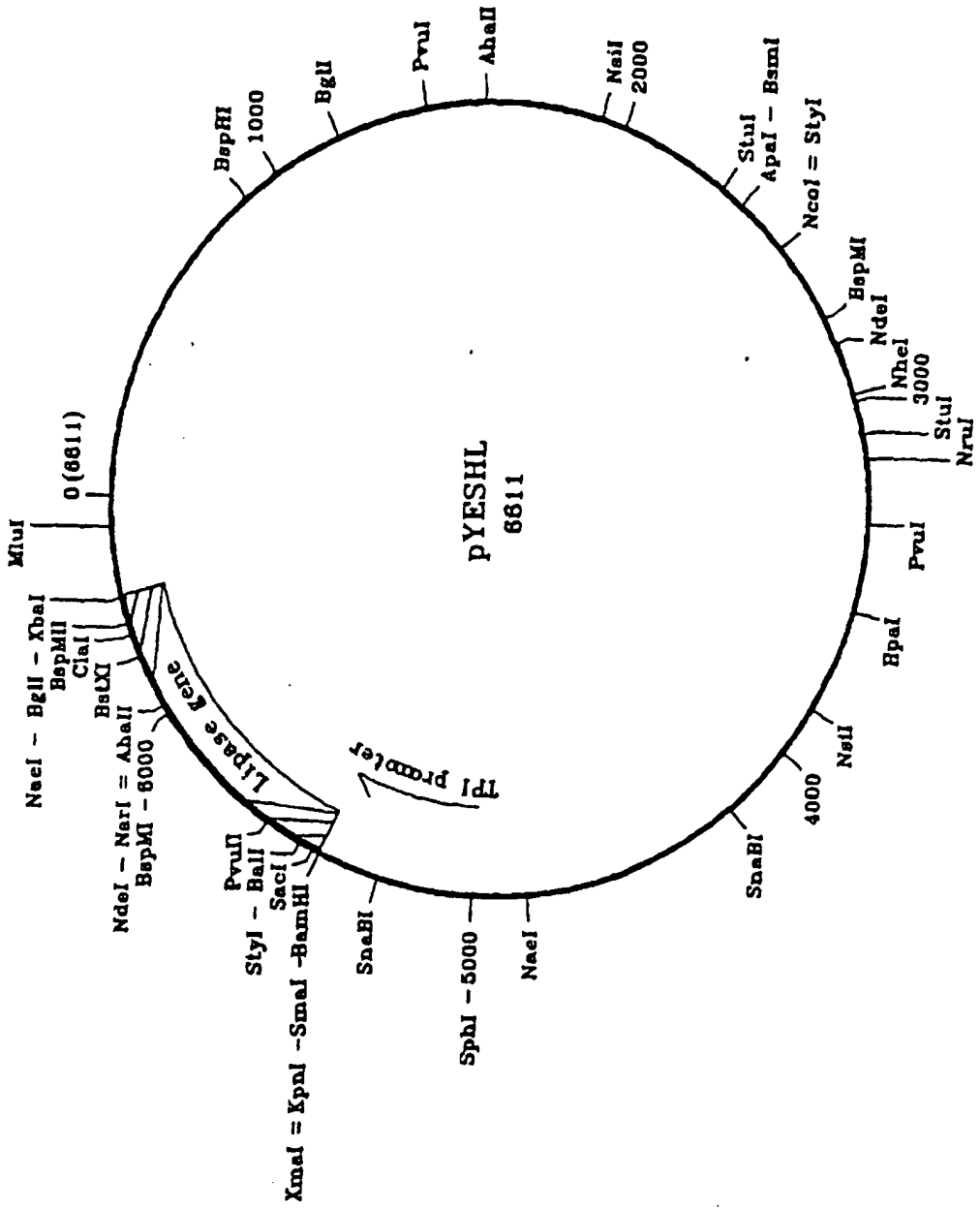
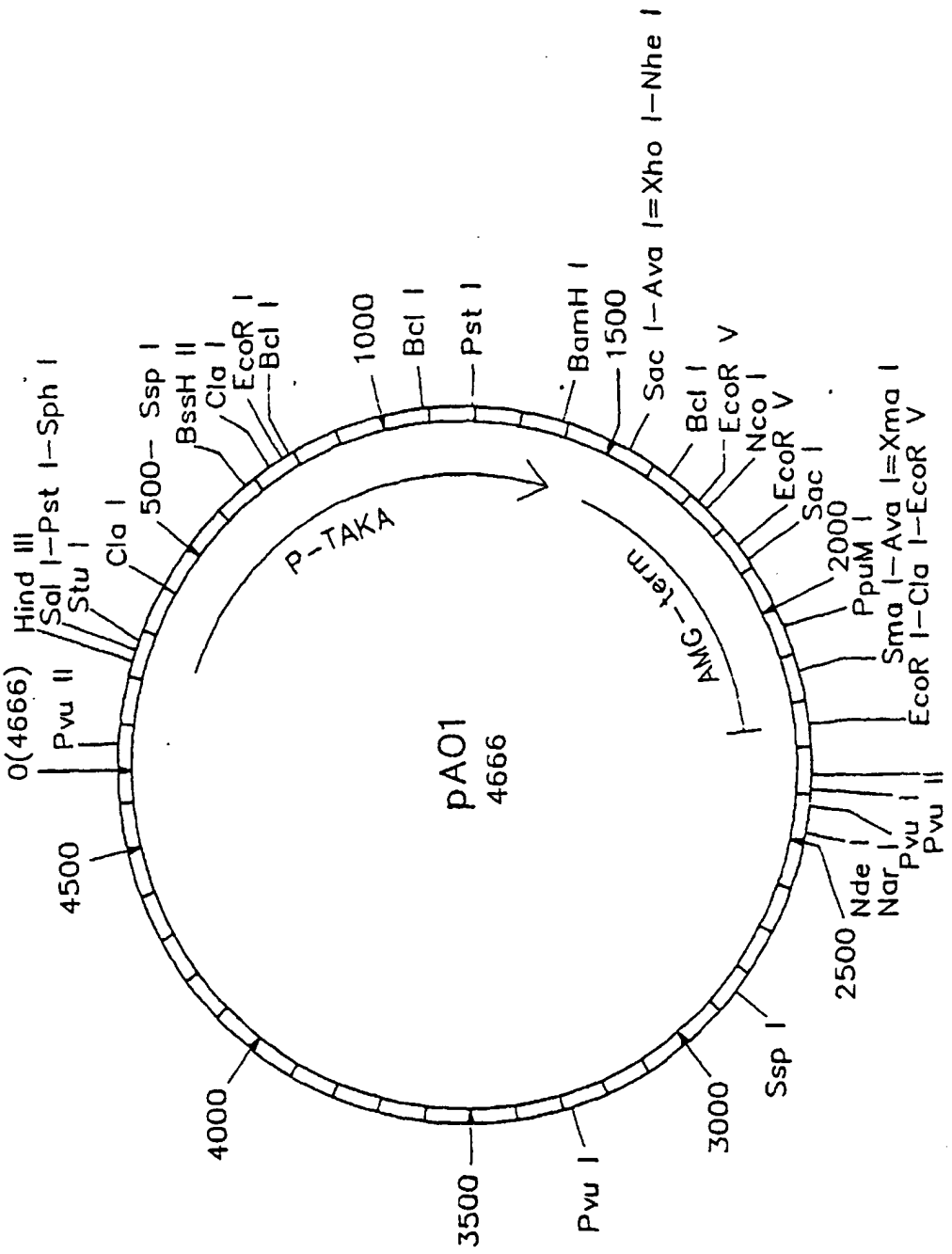

pYESHL
6811
0(6811)
MluI
NaeI - BglII - XbaI
BspMII
ClaI
BstXI
NdeI - NarI = AhaII
BspMI - 6000
PvuII
StyI - BalI
SacI
XmaI = KpnI -SmaI -BamHI
SnaBI
SphI - 5000
NaeI
SnaBI
4000
NsiI
EpaI
PvuI
NruI
StuI
3000
NheI
NdeI
BspMI
NcoI = StyI
StuI
ApaI - BsmI
NsiI
2000
AhaII
PvuI
BglII
1000
BspHI
Lipase Gene
TPI promoter

Fig. 1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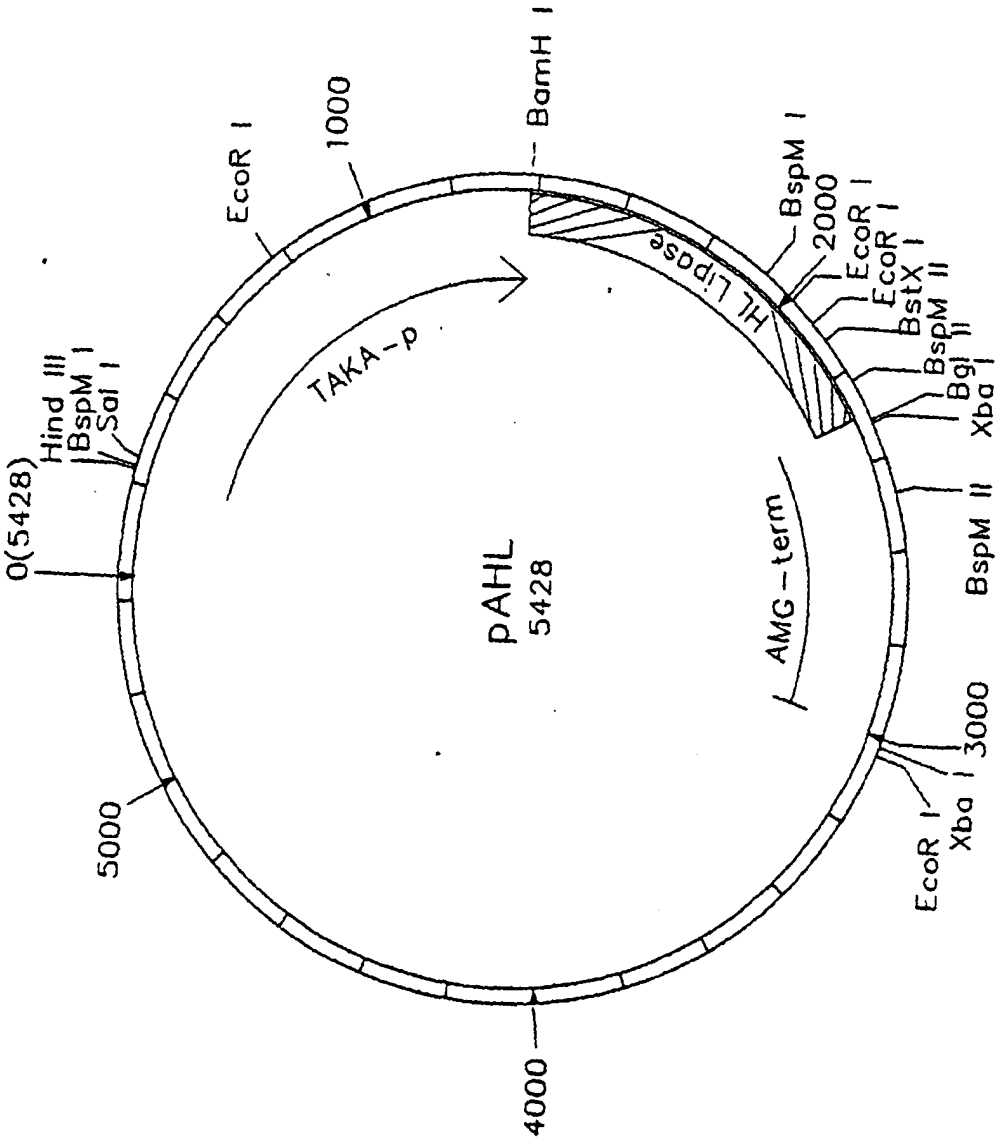
4666
P-TAKA
AMG-term
0(4666)
500
1000
1500
2000
2500
3000
3500
4000
4500
Hind III
Sal I-Pst I-Sph I
Stu I
Pvu II
Cla I
Ssp I
BssH II
Cla I
EcoR I
Bcl I
Bcl I
Pst I
BamH I
Sac I-Ava I=Xho I-Nhe I
Bcl I
EcoR V
Nco I
EcoR V
Sac I
PpuM I
Sma I-Ava I=Xma I
EcoR I-Cla I-EcoR V
Pvu II
Pvu I
Nar I
Nde I
Ssp I
Pvu I

Fig. 2


BamH I
1000
EcoR I
BspM I
2000
EcoR I
EcoR I
BstX I
BspM II
Bgl II
Xba I
HL Lipase
TAKA-p
Hind III
BspM I
Sal I
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pAHL
5428
AMG-term
BspM II
3000
EcoR I
Xba I
5000
4000

Fig. 3

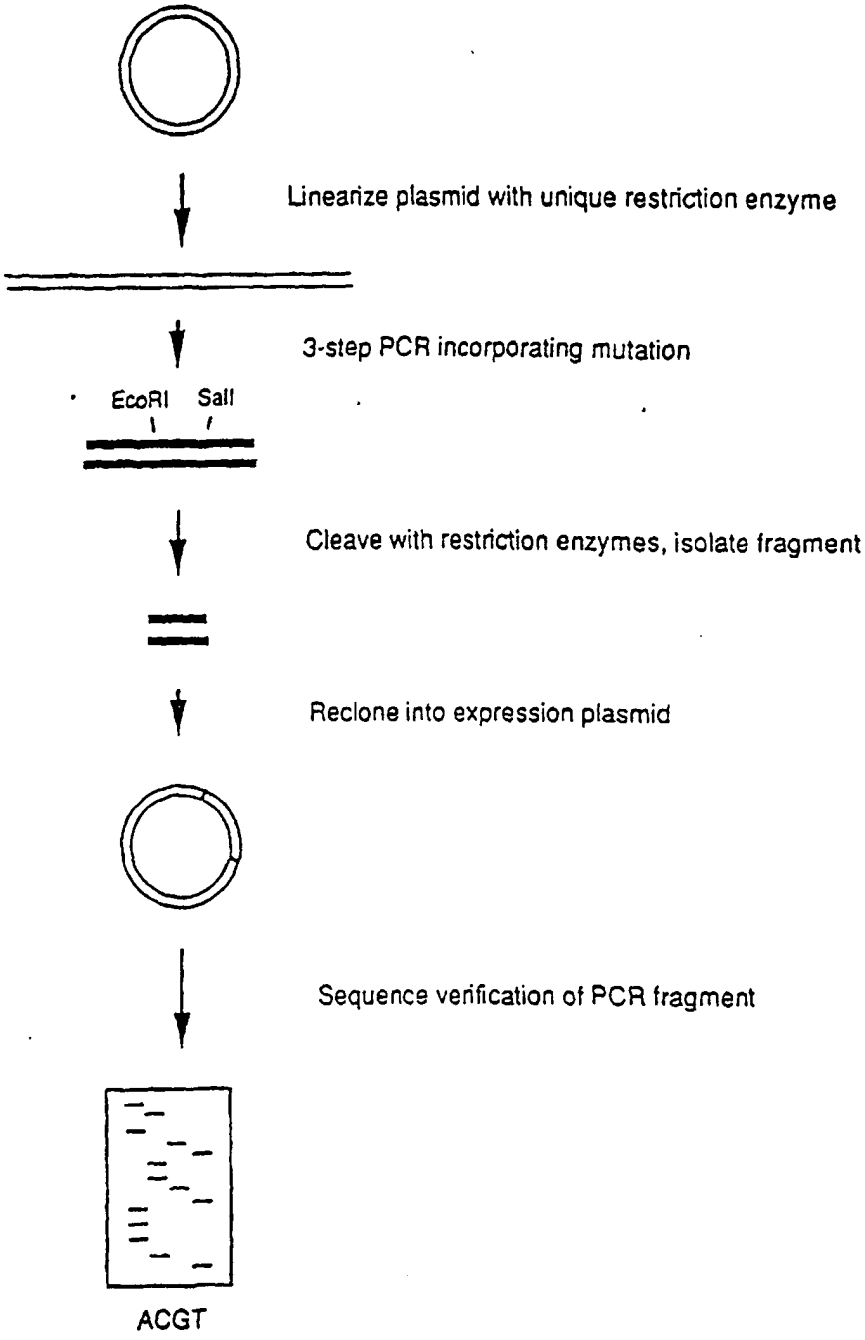
Linearize plasmid with unique restriction enzyme
3-step PCR incorporating mutation
EcoRI
SalI
Cleave with restriction enzymes, isolate fragment
Reclone into expression plasmid
Sequence verification of PCR fragment
ACGT

Fig. 4

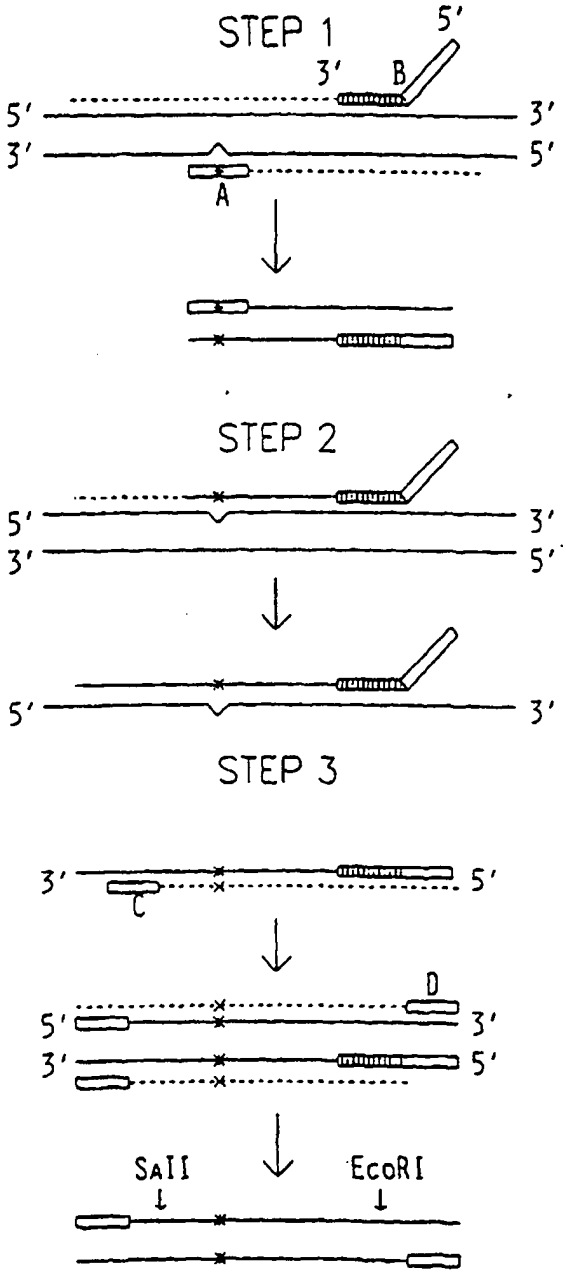

Fig. 5

Application no. JP 07-521525 (1995), Unexamined application no. JP 09-509058 A, Registration no. JP 355395B

CLAIMS

[Claim(s)]

1. It is the Manufacture Approach of Variant of Parent Lipolytic Enzyme. Code of the (a) Parent Lipolytic Enzyme is Carried Out. DNA Array is Left to Random Mutagenesis. Mutagenesis obtained at (b) process (a) A DNA array is discovered in a host cell. It ranks second. Said approach of coming to contain screening to the host cell which discovers the mutagenesis lipolytic enzyme which has the resistance improved to a detergent or a detergent component as compared with the dependency and/or parent lipolytic enzyme which were made to decrease to (c) calcium.

2. the random mutagenesis -- use of physical or a chemical-mutagenesis-ized agent -- or use of an oligonucleotide -- or -- Approach given in the 1st term of a claim performed by use of the PCR creation mutagenesis.

3. Approach given in the 2nd term of claim that mutagenesis-ized agent is chosen from formic acid, UV ****, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulfonate (EMS), sodium hydrogensulfite, and nucleotide analog.

4. Mutagenesis About the manifestation of a DNA array, it is the mutagenesis. It carries out by carrying out the transformation of the suitable host cell which has a DNA array, and it is the mutagenesis. A DNA array is the mutagenesis further by request. A code is carried out in the function permit the manifestation of a DNA array. It changes suddenly coming [a DNA array]. Method given in the 1st term of a claim of cultivating the host cell obtained at the process (b) under the suitable condition for discovering a DNA array.

5. Mutagenesis Approach given in the 1st term of a claim the host cell used in order to make a DNA array discover is a microbial cell.

6. Approach given in the 5th term of claim host cell is cell of fungus or bacterial strain.

7. Host Cell is Group Aspergillus, for Example, A. Nigre, A. ORIZE, and A.
The approach given in the 6th term of a claim which is the cell of the cell of NIDORANSU, or group Saccharomyces, for example, S. SEREBISHIE.

8. approach given in the 6th term of a claim a host cell is gram-positive-bacterium, for example, Bacillus, RIBIDANSU, SUTOREBUTOMAISESU MURINASU, or a gram negative, for example, E. collie. Subtilis and Bacillus RIKENIHORUMISU and Bacillus Wren -- TASS and Bacillus Brevis and Bacillus SUTEARO thermostat philus and Bacillus ARUKARO philus and Bacillus Friend RORIKUEFASHIENSU and Bacillus A KOAGUYU lance and Bacillus A SAKYU lance and Bacillus Rau -- TASS and Bacillus CHU phosphorus diene cis- ** -- streptomyces

9. Approach given in the 1st term of claim mutagenesis lipolytic enzyme has resistance improved to non-ion, anion, cation, zwitter ion, or amphionic surface active agent.

10. A nonionic surface active agent is an alcoholic ethoxy rate, and/or an anionic surface active agent Approach given in the 9th term of a claim which is LAS or alkyl SURUFATO.

11. The method given in the 1st term of a claim of leaving the host cell screened at a process (c) to the second mutagenesis processing, re-screening, re-isolation, and/or re-cloning.

12. Carry out the code of the parent lipolytic enzyme for the random mutagenesis. Approach given in any 1 term of the 1-11th terms of a claim localized in a part of DNA array.
13. An approach given in any 1 term of the 1-12th terms of a claim whose parent lipolytic enzyme is lipase, esterase, KURUCHINAZE, or phospholipase.
14. The nature dialytic ferment of lipophilic is lipase, and the localization random mutagenesis carries out the code of the lipid contact region of parent lipase, or its part. The approach of the claim 12th or 13 term publication performed about a part of DNA array.
15. The localization random mutagenesis carries out the code of a part of the lid (lid) field of parent lipase and/or hydrophobic KUREFUTO (cleft) or this lid field, and/or hydrophobic joint KUREFUTO. Approach given in the 14th term of a claim performed about a part of DNA array.
16. An approach given in any 1 term of the 1-13th terms of a claim to which a parent lipolytic enzyme may originate in a microorganism.
17. The approach given in the 8th term of a claim a parent lipolytic enzyme may originate in a fungus.
18. Carry out the code of the parent lipolytic enzyme. A DNA array is Fumi Kola. sp., RIZOMU call sp., Rhizopus sp., Candida Approach given in the 17th term of a claim which may originate in sp.
19. A parent lipolytic enzyme is lipase and carry out the code of the parent lipase. Approach given in the 18th term of a claim a DNA array is what may originate in the strain of the strain of H. RANUGINOSA, H. RANUGINOSA strain DSM 4109 [for example,], and Rh. Mucor, or the strain of C. anta RUKUCHIKA.
20. the random mutagenesis is entrusted H. RANUGINOSA which a DNA array can obtain from DSM 4109 the amino acid residue 21-27 of lipase, 56-64, and 81-99,108 - 116,145-147,174,202-213,226-227,246-259 or -- 263-269 Approach given in the 19th term of a claim which carries out the code of at least one of the fields pinpointed.
21. The approach given in the 20th term of a claim that the localization random mutagenesis is performed in at least two of these fields.
22. The approach given in the 16th term of a claim a parent lipolytic enzyme may originate in bacteria.
23. Carry out the code of the parent lipolytic enzyme. A DNA array is Pseudomonas. Approach given in the 22nd term of a claim which can be obtained from the strain of a Bacillus from the strain of spp., for example, P. SEPASHIA, P. Alcaligenes, P. SHUDO Alcaligenes, or P. FURAGI.
24. The variant of the lipolytic enzyme manufactured by the approach given in any 1 term of the 1-23rd terms of a claim.
25. It is Variant of H. RANUGINOSA Lipase Which Can be Obtained from DSM 4109, or Its Analog. Mutation in at least one of the locations of a degree : K46, E56, S58, G61, T64, N73, and S -- 83, 190, and G91, N92, N94, D96, L97, K98, E99, I100, D102, A121, E129, D167, R205, E210, K237, N251, I252, D254 and P256 -- G263 and L264 Or variant given in the 24th term of a claim which comes to contain T267.
26. the variant of the analog of H. RANUGINOSA lipase which can be obtained from strain DSM 4109, or this lipase -- it is -- amino acid residue 56-64 and 83-100 or -- 205-211 Said variant which has mutation in at least one of the pinpointed fields.
27. The Following Mutation : K46R, D57G, S58F, G61S, D62C, T64R, S83T, I90F, G91A, N92H, N94I, N94K, L97M, K98I, I100V, D102K, A121V, E129K, D167G, R205K, E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q, or

T267W Variant given in the 26th term of a claim which comes to contain at least one.
N94K+D96A

S83T+N94K+D96N

28. The Following Mutation : E87K+D96V

** -- the variant of H. RANUGINOSA lipase which can be obtained from DSM 4109 which come to contain one even if few, or its analog.
29. Mutagenesis Which Carries Out Code of the Variant of Lipolytic Enzyme Which Has Resistance Improved to Detergent or Detergent Component as Compared with Dependency and/or Parent Lipolytic Enzyme Which were Made to Decrease to Calcium It Comes to Contain DNA Array. It is DNA Construction Object and is **. Process of Approach Given [DNA Array] in Any 1 Term of the 1-23rd Terms of

Claim (C)
The above isolated from the host cell set [is boiled and] and screened DNA construction object.
30. Carry out the code of the H. RANUGINOSA lipase variant of a publication to any 1 term of the 24-28th terms of a claim. DNA construction object.
31. A claim 29th or 30 term publication Vector which has a DNA construction object.
32. The vector given in the 31st term of a claim which is a plasmid or a bacteriophage.
33. Permit the manifestation of the variant of a parent lipolytic enzyme. Vector of the claim 31st or 32 term publication which is the expression vector which comes to contain a DNA array further.
34. A claim 29th or 30 term publication Host cell which has the vector of a publication in any 1 term of a DNA construction object or the 31-33rd terms of a claim.
35. The cell given in the 34th term of a claim which is a microbial cell.
36. The cell given in the 35th term of a claim which is the cell of a fungus or bacterial strain.
37. The cell given in the 36th term of a claim which is the cell of the cell of a group Aspergillus, for example, A. nigre, A. ORIZE, and A. nidulans, or group Saccharomyces, for example, S. cerevisiae.
38. gram positive bacterium, for example, a Bacillus, Subtilis and Bacillus RIKENIHORUMISU and Bacillus Wren -- TASS and Bacillus Brevis and Bacillus SUTEARO thermostat philus and Bacillus ARUKARO philus and Bacillus Friend RORIKUEFASHIENSU and Bacillus A KOAGUYU lance and Bacillus A SAKYU lance and Bacillus Rau -- TASS and Bacillus CHU phosphorus diene cis- ** -- streptomyces RIBIDANSU or streptomyces Cell given in the 36th term of a claim which is MURINASU or a gram negative, for example, E. collie.
39. Said approach of being the approach of manufacturing the variant of the parent lipolytic enzyme which has the dependency over calcium made decreasing, and/or resistance [as opposed to a detergent or a detergent component as compared with a parent lipolytic enzyme], and coming to contain collecting lipolytic enzymes from the host cell which manufactured the variant lipolytic enzyme according to the approach of a publication in any 1 term of the 1-23rd terms of a claim, and was subsequently screened at the process (c).
40. Said approach of be the approach of manufacture the variant of the parent lipolytic enzyme which have the resistance improved to the detergent or the detergent component as compared with the dependency and/or the parent enzyme which be made decrease in number to calcium , and come to contain collect from a culture the variants which cultivated the host cell of a publication in any 1 term of the 34-38th terms of a claim , and be subsequently discovered under the suitable condition for make a variant discover .
41. The variant of the lipolytic enzyme manufactured by the approach a claim 39th or given in 40 terms.
42. The additive for detergents which comes to contain the variant of the lipolytic enzyme of a publication in any 1 term of the claim 24-28th or the 41st term which is in the form of a non-dust nature granulated material, a stabilizer object, or the protected enzyme by request.
43. 0.02-200mg per 1g of additives Additive for detergents given [containing enzyme protein] in the 42nd term of a claim.
44. The claim 42nd which comes to contain other enzyme, for example, protease,

amylase, peroxidases, KUCHINAZE, lipase, and/or cellulases additionally, or the additive for detergents given in 43 terms.
45. The detergent constituent which comes to contain the variant of the lipolytic enzyme of a publication in any 1 term of a claim 24-28th or the 41st term.
46. The detergent constituent given in the 45th term of a claim which comes to contain other enzyme, for example, protease, amylase, peroxidases, KUCHINAZE, lipase, and/or cellulases additionally.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
Field of the manufacture approach invention of the variant of a lipolytic enzyme This invention relates to the variant produced by the manufacture approach of the variant of a parent lipolytic enzyme, and this approach. Furthermore, this invention carries out the code of the variant of this invention. A DNA construction object, ** It is related with the additive for detergents or detergent constituent which comes to contain a variant in the expression vector and host cell list which come to contain a DNA construction object.
Background of invention Over many years, the lipolytic enzyme has been used, sake [as an enzyme for detergents (i.e. remove the dirt of a lipid or a fat from cloth and other fiber)].
For example, various microorganism lipase has been proposed as an enzyme for detergents. Fumi Kola who is indicated by the example of such lipase European Patent (EP)258 068 and EP 305 216 RANUGINOSA lipase, For example, RIZOMU call which is indicated by EP 238 023 MAIHEI lipase, Candida lipase A and B, for example, C. anta RUKUCHIKA lipase, for example, C. anta RUKUCHIKA lipase indicated by EP 214 761 Pseudomonas lipase, for example, P. Alcaligenes, and P. SHUDO Alcaligenes lipase, To EP 218 272, for example, the thing like a publication, P. SEPASHIA lipase, To EP 331 376, for example, the thing like a publication, Bacillus lipase, For example, B. Subtilis lipase (1993, such as dull TOISU), B. SUTEARO thermostat philus lipase (JP 64/744922), and B. PUMIRASU lipase (EP91 00664) are contained.
Furthermore, penicillium which the lipase by which a large number were cloned was indicated and was indicated by 1991, such as a climax grumble and S., at this Jar MUBERUCHI lipase, Geotrichum KANJI dam lipase (1989, such as SHIMADA and Y.) and various Rhizopus lipase (1991, such as Haas and M.J), for example, R. Delmer lipase, R. Rhizopus niveus lipase (KUGIMIYA, W.1992), and R. ORIZE lipase are contained.
In other types of the lipolytic enzyme proposed as an enzyme for detergents, it is international public presentation (WO)88/09367. Pseudomonas which is indicated KUCHINAZE of the MENDOSHINA origin, or (it is a publication to WO 90/09446) fusarium SORANI KUCHINAZE of the origin is contained from PISHI.
The attempt which manufactures the lipase variant which has the property improved to a detergent property in recent years has been made. For example WO 92/05249 The lipase variant which has the improved property was indicated and some properties of a wild type lipase enzyme changed in here, specific, i.e., site specific qualification, of those amino acid sequences. More specifically, the lipase variant was indicated and the amino acid residue beyond one so-called piece or the so-called it of the lipid contact (contact) region of parent lipase was embellished in here.
PCT/DK 93/00225 The lipase variant which has the improved property was indicated

and the amino acid residue which occupies the important location of lipase in here was embellished.
EP 407 225 indicate the lipase variant which was manufactured by qualification of the amino acid specified specifically and which has the resistance improved to a lipolytic enzyme.
EP 260 105 indicated hydrolase and the amino acid residue in 15A was permuted from the active site in here.
Said all lipase variants were built by use of the site-directed mutagenesis which brings about qualification of specific amino acid residue, and this amino acid residue was chosen on the basis of those types based on second [of parent lipase], or those locations within the third structure.
The alternative approach for building the given proteinic mutant or proteinic variant was based on the random mutagenesis. For example, it reaches United States patent (US) 4,898,331. WO 91/01285 Such a technique is indicated.
The need of receiving the new lipolytic enzyme which has washing and/or the dishwashing engine performance which have been improved exists, and the purpose of this invention is manufacturing such an enzyme.
Easy indication of invention Now, this invention person etc. developed the new approach of manufacturing the variant of the lipolytic enzyme which has washing and/or the dishwashing engine performance which have been improved as compared with those parent enzymes. This approach carries out the code of the lipolytic enzyme. It is based on the random or localization random mutagenesis of a DNA array.
In the first field, this invention relates to the manufacture approach of the variant of a parent lipolytic enzyme in more detail. This approach The code of the (a) parent lipolytic enzyme is carried out. A DNA array is left to the random mutagenesis. Mutagenesis obtained at (b) process (a) A DNA array is discovered in a host cell. It ranks second. It comes to contain screening to the host cell which discovers the mutagenesis lipolytic enzyme which has the resistance improved to a detergent, 1, or the detergent component beyond it as compared with the dependency and/or parent lipolytic enzyme which were made to decrease to (c) calcium.
In this invention, it is meant that a phrase "a lipolytic enzyme" shows the enzyme expressing the lipid decomposition capacity like the capacity which decomposes a triglyceride or phospholipid.
A lipolytic enzyme may be lipase, phospholipid, esterase, or KUCHINAZE.
a phrase "the random mutagenesis" is meant in order to show introducing 1 (that is, it being contrasted with site-directed mutagenesis -- as), or the variation beyond it at a conventional method, i.e., the random location of a parent enzyme. Random variation should be embellished. It is typically introduced by exposing much copies of a DNA array to mutagen, and subsequently screening to existence of a variant.
Using it, especially in order that the standard of screening of a process (c) may identify the variant of the parent lipolytic enzyme which has washing and/or the dishwashing property which have been improved as compared with those parent enzymes is taken into consideration.
When a phrase "the dependency made to decrease to calcium" is examined on similar conditions about this invention, in order to show the same activity as the activity of a parent enzyme, meaning needing the calcium of the amount of low is meant.
Probably, the mutation lipolytic enzyme of this invention is substantially independent of existence of calcium in order to show enzyme activity.
A phrase "the resistance improved to a detergent or a detergent component" is meant

of the detergent or detergent component of nearby high concentration from a parent lipolytic enzyme with what means that it is activity.
It is meant about this invention that a phrase "a detergent" shows the mixture of a detergent component usually used to washing or dishwashing. Similarly, it is meant that a "detergent component" shows the component usually found out in a detergent or a dishwashing constituent, and the example is shown in the following publications.
The variant which is obtained by this invention approach in addition to the resistance improved to;, i.e., the dependency made to decrease to calcium, which will be understood as follows and/or a detergent, 1, or the detergent component beyond it shows the lipid decomposition activity of the magnitude which is equal to the lipid decomposition activity of a parent lipolytic enzyme preferably, or exceeds it, when it examines under washing and/or dishwashing conditions.
The screening standard defined by the process (c) of this invention approach can be measured by the suitable well-known approach to this contractor. Especially the suitable assay developed to the purpose of this invention is indicated in the following matter and the knot of an approach.
mutagenesis which carries out the code of the variant of the lipolytic enzyme which has the resistance in which this invention has improved to a detergent or a detergent component as compared with the dependency and/or parent lipolytic enzyme to calcium which were made to decrease in another field It comes to contain a DNA array. a DNA construction object -- being related -- the -- A DNA array is isolated from the host cell chosen at the process (c) of this invention approach.
furthermore, another field -- setting -- this invention the recombination expression vector which has a DNA construction object -- the -- This cell is cultivated under the conditions which support production of a variant in the cell list by which the transformation was carried out by the DNA construction object or its vector, and it is related with the manufacture approach of the variant of the parent lipolytic enzyme which comes to contain collecting variants from a culture medium after an appropriate time.
In the last field, especially this invention relates to the detergent which comes to contain the use of this variant, the additive for detergents, and this variant as an enzyme for detergents in the variant list of a lipolytic enzyme for washing and dishwashing.
Detailed indication of invention The code of the parent lipolytic enzyme is carried out. Cloning of a DNA array The code of the parent lipolytic enzyme which should be left to the random mutagenesis according to this invention is carried out. A DNA array can be isolated from all the cells or microorganisms that produce the target parent enzyme by the well-known approach to this contractor.
For example, it can isolate by establishing cDNA or a genomic library from the organism with which having an array is expected, and subsequently screening to an electropositive clone with a conventional method. The example of such a procedure is a standard method (1989, such as notes and Sambrook).
alike -- following (when array information being able to come to hand) -- the amino acid of a parent enzyme, or (when information cannot come to hand about a parent enzyme) a related lipolytic enzyme -- or -- It is selection of the clone which produces the protein which is the antibody and the reactivity which are produced to the selection and/or the parent lipolytic enzyme of a clone which discover hybridization and/or lipid decomposition, for example, lipase activity, in the oligonucleotide probe manufactured on the basis of a DNA array.
The code of the parent lipolytic enzyme which should be embellished according to

this invention from cDNA or a genomic library is carried out. The desirable method of isolating a DNA array is a parent enzyme. It is based on use of a polymerase chain reaction (PCR) using the oligonucleotide probe which was obtained on the basis of DNA or an amino acid sequence and which degenerated. For example, R.K. SAIKI etc. can perform PCR, using the approach indicated to U.S. Pat. No. 4,683,202 (1988). Alternatively, the code of the parent enzyme is carried out. A DNA array can be manufactured to a resultant by the approach indicated by a phospho aminodite method or MATESU indicated by the standard method, for example, BIKEJI, and cull TERUSU (1981) which were established (1984). By the phospho aminodite method, an oligonucleotide is automatic. With a DNA synthesis machine, annealing is compounded, refined and carried out, and it is combined, and is cloned in a suitable vector.

The code of the parent enzyme is carried out to the last. A DNA array is the genome of a compound die, composition of the synthetic origin and a compound die and the cDNA origin, or the genome of a compound die and the cDNA origin that were prepared by combining the fragment of composition, a genome, or the cDNA origin (in the suitable case) according to a standard method. It is ** to which it can prepare from DNA and a fragment carries out the code of the parent enzyme. It is equivalent to the various parts of a DNA array.

Random mutagenesis The code of the parent lipolytic enzyme which should be performed according to the process (a) of this invention approach is carried out. The random mutagenesis of a DNA array can be conveniently performed to this contractor by the well-known approach.

for example, suitable -- use of physical or a chemical-mutagenesis agent -- use of a suitable oligonucleotide -- or -- DNA array It can carry out by entrusting the PCR creation mutagenesis. Furthermore, use of the combination of the arbitration of these mutagens can perform the random mutagenesis.

A mutagen may induce a rearrangement, transversion, scrambling (scrambling), deletion, and/or insertion.

suitable for this invention -- an ultraviolet-rays (UV) exposure, a hydroxylamine, N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, a nitrous acid, formic acid, and a nucleotide analog are contained in the example of physical or a chemical-mutagenesis agent.

When such drugs are used, typically, the mutagenesis carries out the code of the parent enzyme by which the mutagenesis should be carried out under existence of the mutagen of selection under a suitable condition for the mutagenesis to occur.

Mutation which incubates a DNA array and subsequently has the target property It is carried out by choosing DNA.

When the mutagenesis is performed by use of an oligonucleotide, an oligonucleotide may be doped or spiked by three sorts of non-parent nucleotides during composition of an oligonucleotide in the location where change is desired. Doping or spiking (spiking) is performed so that the codon to non-wanting amino acid may be avoided. The oligonucleotide doped or spiked is PCR, LCR, or arbitration. The code of the lipolytic enzyme is carried out with the announced technique using DNA polymerase and a ligase. It can insert in DNA.

Under the condition which carries out the code of the parent lipolytic enzyme when using the PCR creation mutagenesis and which was processed chemically or increases mistake insertion (misincorporation) of a nucleotide for a non-processing gene PCR is entrusted (1989, such as the day shear 1992 and ROINGU).

E. The mu factor TETA strain of a collie (1974, such as Fowler), S. SEREBISHIE, or

the microorganism organism of other arbitration carries out the code of the lipolytic enzyme by carrying out the transformation of the plasmid containing for example, a parent enzyme to mu factor TETA strain, increasing the mu factor TETA strain which has a plasmid, and subsequently isolating a mutation plasmid from mu factor TETA strain. It can be used to the random mutagenesis of DNA. Then, the transformation of the mutation plasmid is carried out to a manifestation organism.
Mutation should be carried out. A DNA array may be conveniently discovered in the genome prepared from the organism which discovers a parent lipolytic enzyme, or a cDNA library. alternative -- a DNA array -- the suitable plasmid or vector top like a bacteriophage -- it can exist -- these -- the **** incubation -- it can do -- or -- otherwise, it may be exposed to a mutagen. the mutagenesis should be carried out incorporating DNA in the genome of a host cell -- or you may exist in a host cell by existing on the vector introduced into intracellular. The mutagenesis should be carried out to the last. DNA may exist in the form where it was isolated. The random mutagenesis should be entrusted. A DNA array is cDNA or a genome preferably. It is a DNA array.
It is the mutagenesis before a manifestation process (b) or a screening process (c) is performed in some cases. It is convenient to amplify a DNA array. Being able to give this contractor such magnification according to a well-known approach, an approach desirable today is a parent enzyme. The oligonucleotide primer prepared on the basis of DNA or an amino acid sequence is used. It is PCR creation magnification.
It follows being exposed to an incubation or a mutagen with a mutagen, and is mutation. Under the condition which should produce a manifestation for DNA It is discovered by cultivating the suitable cell which has a DNA array. The host cell used to this purpose is mutagenesis which exists on a vector by request. A transformation is carried out in a DNA array, or the code of the parent enzyme is carried out during mutagenesis processing. You may have a DNA array. The example of a suitable host cell is shown below. Mutagenesis A DNA array is the mutagenesis further. The code of the function to permit the manifestation of a DNA array is carried out. It comes to contain a DNA array.
; which is understood as follows and which will come out and exist -- namely, -- The screening standard mentioned at said process (c) was chosen carefully. Therefore, without being restricted to the theory of arbitration, it will be believed that the dependency to calcium made to decrease is what produces the variant which has the engine performance improved on the whole, and the demand to calcium will have the limiting factor to the optimal activity taken into consideration in here under the condition in which a small amount of isolation calcium ion exists especially. In relation to the lipase for detergents, the isolation calcium ion demanded is usually offered from wash water, therefore it depends for lipid decomposition activity on the calcium content of water.
The detergent with which a variant improves resistance, or a detergent component may be the type of arbitration as indicated further at the following. Preferably, a detergent component is non-ion, an anion, a cation, dipolarity, or an amphoteric surface active agent. An alcoholic ethoxy rate is contained in the example of a nonionic surface active agent, and LAS, alkyl sulfate, alcoholic ethoxy sulfate, etc. are contained in the example of an anionic surface active agent.
Especially;, i.e., a nonionic surface active agent alcoholic ethoxy rate, planned as follows and the improved resistance over what can come to hand commercially (the example is Dobanol (trademark)) may be the index of the improved washing engine performance.

Screening of a process (c) is conveniently performed by the use of filter assay based on the following principle. : The microorganism which may discover the target mutagenesis lipolytic enzyme is incubated under suitable conditions to be on a suitable culture medium and secrete an enzyme, and the duplex filter which comes to contain in a culture medium the second filter which shows a low protein binding affinity to the first protein joint filter and its crowning is prepared. A microorganism consists on the second filter. The first filter which comes to contain the enzyme secreted from a microorganism is separated from the second filter which comes to contain a microorganism following an incubation. The corresponding microorganism colony which entrusts the screening for the enzyme activity of a request of the first filter, and exists subsequently to a second filter top is identified.
The filters used in order to combine enzyme activity may be all protein joint filters, such as nylon or a nitrocellulose. The top filters which have the colony of a manifestation organism may be all the filters that do not have the compatibility over proteinic association or have low compatibility, for example, cellulose acetate, and Durapore (trademark). A filter can carry out conditioning on all the conditions that should be used to screening, or can be processed during detection of enzyme activity. Enzyme activity is detectable with other well-known techniques to detection of a color, fluorescence, precipitate, a pH indicator, IR absorbance, or enzyme activity. A detection compound is fixable with the combination of the arbitration of all fixed agent, for example, agarose, agar, gelatin, polyacrylamide, starch, filter paper, and cloth; or a fixed agent.
Lipase activity is detected by the brilliant green, rhodamine B, or the Sudan black combined with a lipid, for example, olive oil, or lard. The screening standard for identifying the variant of the parent lipolytic enzyme which has the improved washing capacity may be the detergent constituent of the arbitration combined with one of EGTA, EDTA, non-ion or anion tenside (tensides), Alkali pH, or said the detection agents of enzyme activity.
;, i.e., the screening standard used by the filter assay of this invention, which will be understood as follows is chosen so that it may respond to the property of a request of an enzyme or use which should be screened. For example, in the screening to the lipase used especially in paper manufacture industry, it is appropriate to screen to the acid lipase which has the temperature stability made to increase. Use of the incubation under the bottom elevated temperature of analysis can perform the buffer solution with which this has Acidity pH (for example, pH4), and/or an analysis front stirrup. Conveniently, the host cell produced at a process (c) can be left more to the round of **** which (Process a)-(c) defined, and also the mutagenesis using a reduction selection standard rather than it is used by previous mutagenesis processing.
The host cell chosen in a process (c) is directly used for manufacture of the variant of a lipolytic enzyme. Alternatively, the code of the variant is carried out. DNA can be isolated from a host cell and may be inserted in other suitable host intracellular by using conveniently the procedure indicated below in the knot (the suitable host cell is also hung up in here) by which the title was carried out to "the manifestation of the variant of this invention."
Localization random mutagenesis According to this invention, the random mutagenesis can be conveniently located in a part of target parent lipolytic enzyme. This is advantageous when it is identified that the fixed field of an enzyme is [as opposed to / especially / the property in which the enzyme was given] important, and when this is embellished, bringing about the variant which has the improved property is expected. By clarifying tertiary structure of a parent enzyme, such a field may

usually be identified, when connected with an enzymic function.
The localization random mutagenesis is the above. It is conveniently performed by other use of a suitable technique well-known to an PCR generating mutagenesis technique or this contractor.
alternatively, it should be embellished The code of a part of DNA array is carried out. a DNA array -- for example, it can isolate by being inserted in a suitable vector, and this part is succeedingly left to the mutagenesis by use of the aforementioned mutagenesis.
Parent lipolytic enzyme The parent lipolytic enzymes which should be embellished according to this invention may be all enzymes that have the **** lipid decomposition activity specified previously. Lipase, esterase, KUCHINAZE, and phospholipid are contained in the example of lipid decomposition activity.
Preferably, a parent lipolytic enzyme carries out the code of a part of lipid contact (contact) zone or this zone. It is embellished by the localization random mutagenesis performed on the part of a DNA array.
All the crystallized lipase that was found out till today comes to contain at least one surface loop structure (called a lid or a flap again) which covers an active site, when lipase is an inactive form (the example of such lipase is indicated by 1990, such as BURAJI). When lipase is activated, loop structure is shifted and it is exposed to active site residue, and a hydrophobic front face is an active site. It is built around Ser and this carries out owner Perilla frutescens (L.) Britton var. crispa (Thunb.) Decne. of the surface hydrophobicity made to increase, and this is hydrolysis or interacts with a lipid substrate during hydrolysis. this activation is called surface activity-ization -- having -- and -- further -- It is discussed by Tilbeurgh etc. (1993).
Or the front face built from activity is called "a lipid contact zone (contact zone)" to the purpose of this invention, and it is the form of loop structure and is located in the part of this front face by request, it is meant that the amino acid residue which forms this part is included. When contact on a lipid front face is activated and lipase hydrolyzes a triglyceride from a lipid, such residue is hydrolysis or can participate in a substrate and a lipase interaction during hydrolysis.
A lipid contact zone contains the joint field to a lipid substrate, and this substrate is the part of the lipid contact zone combined before a single lipid substrate molecule's hydrolyzing. This joint field contains re-**** acyl joint hydrophobic KUREFUTO (creft) and the so-called hydrolysis pocket, and this is an active site. It is located in the surroundings of Ser and it is thought that hydrolysis of a lipid substrate occurs in here. In all the lipase known today, a lipid contact zone is easily recognized from the three-dimensional structure of the lipase built by the suitable computer program, for example. The conformation of inactive and activity lipase is shown in drawing 1 and drawing 2 of WO 92/05249.
Fumi Kola currently discussed in detail with this application The lipid contact zone of RANUGINOSA lipase is amino acid residue 21-25, 36-38, and 81-98,110-116,144-147,172-174,199-213. It reaches. 248-269 It is formed. Such residue was identified on the basis of the computer model simulation of the interaction between lipase and a lipase substrate.
The lipid contact zone of other lipolytic enzymes a) Calculate the hydrophobic vector of the 3-D molecular structure, and a cut (cut) perpendicular to a vector is created through the Calpha-atom of the second amino acid residue after an active site serine within an array b line. Subsequently, all the residue that has at least one atom by the it side of the cut for which c vector is fit is made to contain. subsequently, d -- it is formed by choosing from such residue, and such residue has at least one atom in 5A

of a proteinic (in the case of the lipase in the form opened or closed) front face.
In the case of the lipase in the form opened or closed, a hydrophobic vector is calculated from protein by totaling all residue vectors to the residue which has at least 10% of surface accessibility (Lee, B. and Richard, F.M.1971, Mol.Blol.55:379-400). The starting point of a residue vector is specified as a Calpha-atom of residue, and the direction passes along the center of mass of a side chain. Each residue magnitude of a vector is specified as relative transfer free energy of residue.
The surface accessibility of each residue is calculated using a KONNORI (Connolly) program.
Preferably, the localization random mutagenesis carries out the code of a part of the lid (lid) field of parent lipase and/or hydrophobic KUREFUTO or this lid field, and/or hydrophobic KUREFUTO. It is carried out on the part of a DNA array.
The parent lipolytic enzyme which should be embellished according to this invention may be the thing of all the origins. Therefore, an enzyme may be the field origin of mammalian, vegetation, a vertebrate, or other arbitration. However, it is built from the microorganism origin, and much microorganism strain is found out, and an enzyme produces the enzyme of a specific application to the purpose for detergents today. Furthermore, specifically, a DNA array parent lipolytic enzyme may originate in a fungus, i.e., yeast, or a fibrous fungus. For example, a DNA array is Fumi Kola. sp., for example, strain of H. RANUGINOSA, RIZOMU call The strain of sp., for example, Rh. MAIHEI, Rhizopus Strain of sp., Candida The strain of sp., FUSARIAMU sp., for example, F. SORANI, Strain of PISHI, You may be what can originate in the strain of the strain of the strain of Benz rear spp., for example, V. INEKU Alice, KORETOTORIKUMUspp., for example, C. GUROE loss poly ROIDESU, or C. RAGENARIUMU, penicillium spp., for example, P. SUPINUROSAMU, or P. jar MUBERUCHI.
this invention -- setting -- "-- since -- it can originate -- " -- the enzyme produced by the strain of the target organism is not only shown, but it was isolated from such an enzyme a code is carried out according to a DNA array -- having -- and ** The enzyme produced within the host organism by which the transformation was carried out in the DNA array is also shown. Furthermore, the phrase is composition and/or the cDNA origin. A code is carried out by the DNA array and it is meant that the enzyme which has the description which identifies the target enzyme is shown.
Especially an interesting thing is lipase originating in the analog of H. RANUGINOSA, for example, the lipase originating in the strain of H. RANUGINOSA strain DSM 4109, or this lipase, Rh. phycomycosis stock, or the strain of C. anta RUKUCHIKA as a parent lipolytic enzyme.
Setting to this invention, only for one piece or the amino acid residue beyond it, a phrase "an analog" is H.
It comes to contain a different amino acid sequence from it of RANUGINOSA lipase (measuring as extent of identity between two arrays). It is the amino acid sequence of this lipase, at least 70% of homology, for example, at least 75%, 80%, 90%, or 95% homology. It is cross-reactivity as immunologically as this lipase, and/or the code of the amino acid sequence of this lipase or this lipase is carried out. Hybridization is carried out to the oligonucleotide probe prepared on the basis of the DNA array. According to a DNA array It is meant that the polypeptide by which a code is carried out is included.
An analog one piece or the amino acid residue beyond it The addition to both the amino terminal of lipase, and both [one side or], By different part beyond one piece or it in an amino acid sequence, one piece or the permutation of the amino acid

residue beyond it, They are one of the two or the both ends of lipase. By the part beyond one piece or it in an amino acid sequence One piece or the deletion of the amino acid residue beyond it, Or the code of the lipase to which it carries out raw [of the insertion of the amino acid residue beyond one piece or it] by the part beyond one piece or it in an amino acid sequence is carried out. You may be the derivative of H. RANUGINOSA lipase prepared by embellishing a DNA array. The combination of these techniques according to a site specific, the random mutagenesis, or a well-known technique can perform qualification of a DNA array.
Furthermore, an analog may be a polypeptide originating in other organisms like one of the organisms mentioned in the knot of the aforementioned "background of invention."
The code of the analog of the parent H. RANUGINOSA lipase using the oligonucleotide probe of relation is carried out. The hybridization of a DNA array can perform a DNA array under the suitable conditions which carry out hybridization. For example, such conditions are for example, 5xSSC. The pre soaking and 20% formamide in inside, 5x Denhardt's solution, the sodium phosphate of 50mM (pH 6.8), And cow thymus gland by which denaturation sonication of the 50microg was carried out Among the solution of DNA, at -40 degree C 1-hour reserve hybridization, It ranks second. The hybridization under the specific conditions which contain hybridization at -40 degree C for 18 hours in this solution which added 100microM ATP, or Sambrook(s) are other approaches indicated by 1989.
H. The immunological cross reaction of the analog of RANUGINOSA lipase is analyzed using it and a reactant antibody, or it was generated to at least one epitope of the refined lipase. The antibody which may be any of a monoclonal or a polyclonal is manufactured by the well-known approach by this contractor indicated by Hudson etc. An immunological cross reaction is measured by this contractor using well-known analysis, and the example is radiation immunity analysis, as indicated in 1989, such as waste turn blotting (Western Blotting) or Hanson.
When a parent lipolytic enzyme is H. RANUGINOSA lipase which can be obtained from strain DSM 4109, or its analog, The following contents are left, desirable;, i.e., random mutagenesis. A DNA array the amino acid residue 21-27 of this lipase, and 56- 64 and 18 -99 and 83-100,108-116,145-147,174,202-213 -- for example, -- 205-211,226-227,246-259 -- or -- 263-269 The code of at least one of the fields regulated is carried out. The part is constituted coming [a part of DNA array]. This lipase DNA and an amino acid sequence are clear from the array numbers 1 and 2 respectively.
The localization random mutagenesis can be performed [more than one or it of these fields], and is preferably performed in at least two fields.
The parent lipolytic enzyme which should be embellished according to this invention can originate in bacteria. For example, the code of the parent lipolytic enzyme is carried out. A DNA array is Pseudomonas spp., for example, P. SEPASHIA, P. Alcaligenes, P. shoe door RUKARIGENSU, and P.
The strain or the streptomyces of the strain of MENDOSHINA (called P. PUCHIDA again), P. syringe, P. EROGINOSA, or P. FURAGI, Bacillus spp., for example, B. Subtilis, or B. PUMIRASU It can originate in the strain of sp., for example, S. SUKABI.
A parent bacteria lipolytic enzyme is the Pseudomonas lipase like a publication, or international public presentation (WO)88/09367 at the lipase of said seed origin, EP 218 272 [for example,], EP 331 376, and EP 407 225. You may be KUCHINAZE which is indicated.

Variant of this invention In order to make reference easy, the specific variant of this invention is indicated by use of the next naming. : The amino-acid (1 or more):location which is a basis (1 or more): Permutation amino acid (1 or more) This naming is followed. permuting a valine by the aspartic acid in a location 96 is shown as following : Asp 96 Val or -- D96V The deletion of an aspartic acid is shown by the same location as following. : Asp 96 * or -- D96* And additional amino acid residue, for example, insertion of a lysine, is shown as following : Asp 96 ValLys or -- D96VK multiplex variation is separated by plus -- namely : Asp 96 Val+Glu 87 Lys or -- D96 V+E87K The variation which permutes a valine and a lysine with an aspartic acid and glutamic acid in locations 96 and 87, respectively is shown.
the time of the alternative amino acid residue beyond 1 or it being inserted in the given location -- it D96V and N or -- It is shown as D96V or D96N.
Furthermore, the suitable location for qualification should be understood as follows, when identified without any proposed specific qualification in this invention.;
That is, amino acid residue can be permuted by the amino acid residue which exists in a location. Therefore, also although it is not clearly shown for example, when qualification of the aspartic acid of a location 96 is mentioned Deletion of the;, i.e., aspartic acid, which should be understood as follows may be carried out, or it may be permuted by other amino acid (any one [i.e.,] of R, N, A, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, and the V), or the amino acid inserted further in the location.
finally, the variation of parent H. RANUGINOSA lipase is identified by this detail letter, and should be understood as a thing including the same variation of the analog of this (**** defined previously) lipase.
In another field, this invention relates to the variant built by said approach of this invention.
When a parent lipolytic enzyme is H. RANUGINOSA lipase which can be obtained from strain 4109, or the analog of ****** defined previously, a variant comes to contain variation in at least one location of the following location:S58, T64, S83, N94, K98, I100, A121 and E129, D167P R205, K237, I252 and P256, or G263. It is the amino acid residue which the amino acid residue of arbitration other than wild type amino acid residue is inserted the case of;, i.e., a permutation, which will be understood as follows, for example, is chosen from R, N, A, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, V, and D.
As far as this invention gets to know, the conventional indication of the specific variation in these locations does not exist.
In addition, in here, amino acid residue L264 was permuted by any one of the different amino acid from a leucine, i.e., R, N, A, C, Q, E, G, H, I, K, M, F, P, S, T, W, Y, V, and D, about the variant of H. RANUGINOSA lipase which can obtain this invention from DSM 4109, or the analog of this lipase.
The variant concerning this invention preferably The following mutation K46R, E57G, G61S, S83T, S58F, D62C, T64R, I90F, G91A, N92H, N94I, N94K, L97M, K98I, I100V, D102K, A121V, E129K, D167G, R205K, E210W, It comes to contain K237M, N259W, I252L, D254W, P256T, G263A, L264Q, or at least one of the T267W.
It plans to have found out enzyme activity and/or detergent resistance with these locations important to them. Numbering of amino acid residue makes reference in the amino acid sequence of mature lipase.
Preferably, the variant concerning this field of this invention is the following mutation. It comes to contain at least one of S83T, N94K, A121V, D167G, and the R205K.

mutation beyond 1 or it defined by the combination or this specification beyond 1 or it of the mutation as which it comes out and;, i.e., this invention, which is understood as follows, and which will exist is defined by this detail letter WO 92/05249 and WO 94/25577 -- and -- WO 94/01541 The variant of the parent H. RANUGINOSA lipase which comes to contain the combination of the mutation of the arbitration indicated is included.

Furthermore, it sets to another field. This invention The following mutation : N94 K+D96AS83 T+N94 K+D96NE87 K+D96VE87 K+G91 A+D96AN94 K+F95 L+D96HA121 V+R205 K+E210QF95 C+D96NG91 S+L93 V+F95CE87 K+G91 A+D96 R+I100VE87K + G91AS83 T+E87 K+Q249RS83 T+E87 K+W89 G+G91 A+N94 K+D96VN73 D+S85 T+E87 K+G91 A+N94 K+D94AE87 K+G91 A+L93 I+N94 K+D96AD167 G+E210VN73 D+E87 K+G 91 A+N94 I+D96GS83 T+E87 K+G91 A+N92 H+N94 K+D96ME210WE56 T+D57 L+I90 F+D96 L+E99KE56 R+D57 L+V60 M+D62 N+S83 T+D96 P+D102ED57 G+N94 K+K96 L+L97 ME87 K+G91 A+D96 R+I100 V+E129 K+K237 M+I252 L+P256 T+G263 A+L264QE56 R+D57 G+S58 F+D62 C+T64 R+E87 G+G91 A+F95 L+D96 P+K98 I+K237MK46 R+E56 R+G6
1SD102KD167GN73D+E87K+G91A+N94I+D96GE210VE210WN251W+D254W+ T267WS83T+E87K+G91A+N92H+N94K+D96ME56R + I90 F+D96 L+E99KD57 G+N94 K+D96 L+L97M It is related with the variant of H. RANUGINOSA lipase which can be obtained from DSM 4109 which come to contain at least one, or its analog.

It is found out that the improved resistance over the resistance and/or the detergent component, for example, the nonionic surface active agent alcoholic ethoxy rate, made to decrease to calcium is shown, and these variants follow, and are especially considered to be useful to the purpose of a detergent or dishwashing. A variant is built by this invention approach and indicated by property-izing and the example of further the following about the mutation introduced succeedingly. The alternative approach of building;, i.e., these variants, which will be clear has set the foundation to site-directed mutagenesis using the suitable oligonucleotide probe. This approach is explained by Example 3-6.

Manifestation of the variant of this invention Mutagenesis which carries out the code of the variant enzyme manufactured by said approach or the alternative approach well-known to this contractor according to this invention A DNA array may be typically discovered in an enzyme form using the expression vector in which at least a promotor, an operator, and a ribosome bond part contain a repressor gene or various activation genes by the transfer start signal and request.

The code of the variant of this invention is carried out. The recombinant expression vector which has a DNA array is recombinant. You may be all the vectors conveniently left to a DNA procedure, and it will often depend for selection of a vector on the host cell into which it should be introduced. Therefore, a vector is an autonomous duplicate vector, i.e., the vector which exists as actual existence chromosome outside, for example, a plasmid, a bacteriophage or the element outside a chromosome, a minichromosome, or an artificial chromosome. Alternatively, when introduced into a host cell, a vector may be included in a host cell genome and may be reproduced with the chromosome in which it was included.

In the vector, the DNA array should be connected to the suitable promotor array operational. A promotor is arbitration which shows transcriptional activity in the host cell of selection. You may be a DNA array and it can originate in the gene which carries out the code of the protein of homologous or an ununiformity to a host cell.

The example of the suitable promotor for ordering especially a bacteria host's imprint E. Collie The promotor of the lac operon, streptomyces KOERI color agarose gene dagA promotor, Bacillus The promotor of a RIKENIHORUMISU alpha-amylase gene (dymL), For example WO 93/10249 The thing like a publication, Bacillus The promotor of a SUTEARO thermostat philus maltose production amylose gene (amyM), The promotor of Bacillus friend RORIKUEFASHIENSU alpha-amylase (amyQ), Bacillus They are promotors, such as Subtilis xylA and a xylB gene. A useful promotor's example is an A. ORIZE TAKA amylase and a RIZOMU call to an imprint in a fungus host. It originates in the gene which carries out the code of MAIHEI aspartic protease, A. nigre neutral alpha-amylase, A. nigre acid stability alpha-amylase, A. nigre glucoamylase, Rhizomucor miehei lipase, A. ORIZE alkaline protease, A. ORIZE triosephosphate isomerase, or A. nidran SUASETAMIDAZE. The expression vector of this invention carries out the code of the variant of this invention into a suitable imprint terminator and the Shinsei living thing again. The polyadenylation array connected to the DNA array operational can be included. A halt and a polyadenylation array can originate suitably from the same origin as a promotor. A vector makes a vector reproduce in the target host cell further. It comes to contain a DNA array. The example of such an array is the origin of the duplicate of plasmids pIJ [pUC19, pACYC177, pUB110, pE194, pAMB1, and] 702.
A marker with a vector selectable again, for example, the product of ********, supplements with a host intracellular deficit, or it comes to contain what gives the antibiotic resistance like ampicillin, a kanamycin, a chloramphenicol, or tetracycline resistance. Furthermore, a vector comes to contain the marker which produces an Aspergillus selective marker, for example, amdS, argB, nidD and sC, and hygromycin tolerance, or selection is WO 91/17243. The cotransformation like a publication can attain.
For example, when using a certain bacteria as a host cell, although the intracellular manifestation is advantageous in some respects, generally it is desirable that a manifestation is an extracellular cell. A parent lipolytic enzyme can include the reserve field which makes a culture medium secrete the enzyme discovered in itself. When desirable, a different reserve field or a different signal sequence can permute this reserve field, and it carries out the code of each reserve field. It is conveniently attained by the permutation of a DNA array.
the example of suitable bacteria is gram-positive-bacterium, for example, Bacillus, RIBIDANSU, streptomyces MURINASU, or a gram negative, for example, E. collie. Subtilis and Bacillus RIKERUHORUMISU and Bacillus Wren -- TASS and Bacillus Brevis and Bacillus SUTEARO thermostat philus and Bacillus ARUKARO philus and Bacillus Friend RORIKUEFASHIENSU and Bacillus A KOAGUYU lance and Bacillus A SAKYU lance and Bacillus Rau -- TASS and Bacillus Megger TERIUMU and Bacillus CHU phosphorus diene cis- ** -- streptomyces a bacterial transformation -- for example, a protoplast transformation -- or the very thing -- it can carry out using a competent cell by the well-known approach.
A yeast organism is Saccharomyces or the Schizosaccharomyces kind, for example, Saccharomyces. It can choose from SEREBISHIE preferably. A fibrous fungus is an Aspergillus kind, for example, an Aspergillus, conveniently. ORIZE, Aspergillus Nigre or Aspergillus It belongs to NIDORANSU. a fungus cell -- protoplast formation and the transformation following ** of a protoplast -- the very thing -- a transformation may be carried out by the process which includes playback of a cell by the well-known approach. The suitable procedure for the transformation of an Aspergillus host cell is indicated by EP 238 023.

Furthermore, in another field, it comes to contain this invention cultivating a host cell like the above under the condition on which this approach supports production of a variant about the manufacture approach of the variant of the parent lipolytic enzyme of this invention, and collecting variants from a cell and/or a culture medium subsequently.

The culture medium used in order to cultivate a cell may proliferate the target host cell, and may be all the usual culture media suitable in order to obtain the manifestation of the parent lipolytic enzyme of this invention. or [that a suitable culture medium can come to hand from the provider on commerce] -- or it can manufacture according to the proclaimed process (setting to the catalog of for example, an American type culture collection).

The variant of this invention secreted from a host cell separates a cell from a culture medium by centrifugal separation or filtration, and is recovered from a culture medium by the procedure of the common knowledge which the protein component of a culture medium is settled using a salt, for example, an ammonium sulfate, and subsequently contains **** chromatography methods, such as an ion exchange chromatography and an affinity chromatography.

The additive for detergents and constituent for dishwashing and washing Especially the variant conforms good especially to the execution to the detergent constituent which has activation planned within the limits of pH 8-11 within the limits of the detergent constituent 7-13, for example, pH, for the dependency made to decrease to the calcium to the detergent or detergent component of this invention, and/or the improved resistance.

Detergent constituent According to this invention, the lipase variant of this invention is the component of a detergent constituent typically. By the ****, it may be contained in a detergent constituent in the form of a non-dust nature granulated material, a stabilizer object, or the protected enzyme. a non-dust nature granulated material -- for example, United States patent it is indicated by 4,106,991 and 4,661,452 (both sides give the Novo Indus tree) -- as -- it can manufacture -- and a request -- the very thing -- it can cover with a well-known approach. the example of a wax coating ingredient -- mean molecular weights 1000-20000 Pori (ethylene oxide) product (polyethylene-glycol, PEG); which it has -- ethoxyl-ized nonyl phenol; which has 16 - 50 ethylene-oxide unit -- it is Monod, the G, and the triglyceride of the ethoxyl-ized fatty alcohol; fatty alcohol; fatty-acid; and the fatty acid with which alcohol carries out owner Perilla frutescens (L.) Britton var. crispa (Thunb.) Decne. of the 12-20 carbon atoms, and 15-80 ethylene oxide units exist. The example of the film formation coating matter which fitted application with the fluidized bed process is the British patent. It is shown in 1483591. A liquid enzyme preparation is stabilized by, for example, adding polyol, for example, propylene glycol, sugar or sugar-alcohol, a lactic acid, or a boric acid according to the established approach. Other enzyme stabilizers are common knowledge at this contractor. The protected enzyme can be manufactured according to the approach indicated by EP 238,216.

The detergent constituent of this invention may exist as all convenient gestalten, for example, powder, granulation, a paste, or a liquid. or [that liquid detergent is aquosity which contains the water to 70%, and 0-30% of organic solvent typically] -- or you may be non-aqueous.

The each of a detergent constituent may be an anion, non-ion, a cation, or dipolar ion coming [one sort or the surface active agent beyond it]. Probably, the detergent usually contains anionic surface active agent [0-50% of] (LAS), for example, linear-alkyl-benzene sulfonate, alpha olefin sulfonate (AOS), alkyl SURUFATO (fatty

alcohol SURUFATO), second alkane sulfonate (SAS), alpha-sulfo fatty-acid methyl ester, and alkyl -, an alkenyl succinic acid, or soap. A detergent may contain 0-40% of nonionic surface active agent (AEO or AE), for example, alcoholic ethoxy RATO, carboxylation alcoholic ethoxy RATO, nonyl phenol ethoxy RATO, alkyl poly glycoside, an alkyldimethyl amine oxide, ethoxyl-ized fatty-acid monoethanolamide, fatty-acid monoethanolamide, or a polyhydroxy alkyl fatty-acid amide (it is a publication to WO 92/06154) again.
A detergent constituent may also contain one or more sorts of other enzymes, for example, an amylase, pullulanase, KUCHINAZE, a protease, a cellulase, a peroxidase, an oxidase, and/or (for example, laccase) other lipase additionally.
A detergent may contain 1-65% of detergent builder or complexing agent, for example, zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), and alkyl - or an alkenyl succinic acid, fusibility silicate, or stratified silicate (for example, SKS-6 from Hoechst A.G.). A detergent may not be builder combination, namely, does not have a detergent builder in essence.
The example in which a detergent may also contain one or more polymers is a carboxymethyl cellulose (CMC), Pori (vinyl pyrrolidone) (PVP), a polyethylene glycol (PEG), Pori (vinyl alcohol) (PVA), poly carboxylate, for example, polyacrylate, a maleic acid / acrylic-acid copolymer, and lauryl methacrylate / acrylic-acid copolymer.
A detergent can contain the bleaching system which may also contain 2OH2 source, for example, perborate, or par carbonate, and this can be combined with a peroxy-acid formation bleaching activator (TAED), for example, tetraacetylethylendiamin, or a nonanoyl oxybenzene sulfonate (NOBS). Alternatively, a bleaching system may also contain an amide, imide, or sulfonate type peroxy acid.
the enzyme of the detergent constituent of this invention -- a stabilizer, for example, polyol, for example, propylene glycol, or glycerol, sugar or sugar-alcohol, a lactic acid, a usual boric acid, or a usual boric-acid derivative, for example, aromatic series borate ester, -- using -- it can stabilize -- and a constituent -- for example, -- WO 92/19709 and -- WO 92/19708 It may be pharmaceutical-preparation-ized like a publication.
A detergent can contain the textile softening agent containing other the usual detergent components, for example, clay, a foam formation improver, a soil inhibitor, a **-proof agent, a soil-suspending agent, a color, an anti-soil-reattachment agent, a germicide, a fluorescent brightener, or perfume.
(It was measured in the aquosity solution by operating concentration) Probably, pH consists in neutrality or alkalinity, for example, within the limits of 7-11.
Even if few [come / the component below : (1) by which the following are contained in the specific gestalt of the detergent constituent of this invention within the limits /]
Detergent constituent blended as a granulated material which has the bulk density of 600 g/l

直鎖アルキルベンゼンスルホナート（酸として計算）	7－12%
アルコールエトキシスルファート（例えばC_{12-18}アルコール、1-2 EO）又はアルキルスファート（例えばC_{16-18}）	1 － 4 %
アルコールエトキシラート（例えばC_{14-15}アルコール、7 EO）	5 － 9 %
炭酸ナトリウム（Na_2CO_3として）	14－20%
可溶性シリケート(Na_2O, $2SiO_2$として）	2 － 6 %
ゼオライト（$NaAlSiO_4$として）	15－22%
硫酸ナトリウム（Na_2SO_4として）	0 － 6 %
クエン酸ナトリウム／クエン酸（$C_6H_5Na_3O_7$／$C_6H_8O_7$として）	0 －15%
ナトリウムパーボラート($NaBO_3 \cdot H_2O$ として）	11－18%
TAED	2 － 6 %
カルボキシメチルセルロース	0 － 2 %
ポリマー（例えばマレイン酸／アクリル酸コポリマー、PVP, PEG）	0 － 3 %
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば土壌抑制剤、香料、螢光増白剤、フォトブリーチ）	0 － 5 %

(2) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

直鎖アルキルベンゼンスルホナート（酸として計算）	6－11%
アルコールエトキシスルファート（例えばC_{12-18}アルコール、1-2 EO）又はアルキルスファート（例えばC_{16-18}）	1－3%
アルコールエトキシラート（例えばC_{14-15}アルコール、7 EO）	5－9%
炭酸ナトリウム（Na_2CO_3として）	15－21%
可溶性シリケート（Na_2O, $2SiO_2$として）	1－4%
ゼオライト（$NaAlSiO_4$として）	24－34%
硫酸ナトリウム（Na_2SO_4として）	4－10%
クエン酸ナトリウム／クエン酸（$C_6H_5Na_3O_7$／$C_6H_8O_7$として）	0－15%
カルボキシメチルセルロース	0－2%
ポリマー（例えばマレイン酸／アクリル酸コポリマー、PVP, PEG）	1－6%
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば土壌抑制剤、香料）	0－5%

(3) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

直鎖アルキルベンゼンスルホナート（酸として計算）	5－9％
アルコールエトキシラート（例えばC_{12-15}アルコール、7 EO）	7－14％
脂肪酸としての石けん（例えばC_{16-22}脂肪酸）	1－3％
炭酸ナトリウム（Na_2CO_3として）	10－17％
可溶性シリケート（Na_2O, $2SiO_2$として）	3－9％
ゼオライト（$NaAlSiO_4$として）	23－33％
硫酸ナトリウム（Na_2SO_4として）	0－4％
ナトリウムパーボラート（$NaBO_3 \cdot H_2O$ として）	8－16％
TAED	2－8％
ホスホナート（例えばEDTMPA）	0－1％
カルボキシメチルセルロース	0－2％
ポリマー（例えばマレイン酸／アクリル酸コポリマー、PVP, PEG）	0－3％
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1％
少量成分（例えば土壌抑制剤、香料、螢光増白剤（	0－5％

(4) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

直鎖アルキルベンゼンスルホナート（酸として計算）	8－12％
アルコールエトキシラート（例えばC_{12-15}アルコール、7 EO）	10－25％
炭酸ナトリウム（Na_2CO_3として）	14－22％
可溶性シリケート（Na_2O, $2SiO_2$として）	1－5％
ゼオライト（$NaAlSiO_4$として）	25－35％
硫酸ナトリウム（Na_2SO_4として）	0－10％
カルボキシメチルセルロース	0－2％
ポリマー（例えばマレイン酸／アクリル酸コポリマー、PVP, PEG）	1－3％
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1％
少量成分（例えば土壌抑制剤、香料）	0－5％

(5) The aquosity liquid detergent constituent which comes to contain the following components

直鎖アルキルベンゼンスルホナート（酸として計算）	15－21％
アルコールエトキシラート（例えばC_{12-15}アルコール、7 EO又はC_{12-15}アルコール、5 EO）	12－18％
脂肪酸としての石けん（例えばオレイン酸）	3 －13％
アルケニルコハク酸（C_{12-14}）	0 －13％
アミノエタノール	8 －18％
クエン酸	2 － 8 ％
ホスホナート	0 － 3 ％
ポリマー（例えばPVP，PEG）	0 － 3 ％
ボラート（B_4O_7として）	0 － 2 ％
エタノール	0 － 3 ％
プロピレングリコール	8 －14％
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1％
少量成分（例えば分散剤、土壌抑制剤、香料、蛍光増白剤）	0 － 5 ％

(6) The liquid detergent constituent built by the aquosity which comes to contain the following components

直鎖アルキルベンゼンスルホナート（酸として計算）	15－21%
アルコールエトキシラート（例えばC_{12-15}アルコール、7 EO、又はC_{12-15}アルコール、5 EO）	3 － 9 %
脂肪酸としての石けん（例えばオレイン酸）	3 －10%
ゼオライト（$NaAlSiO_4$として）	14－22%
クエン酸カリウム	9 －18%
ボラート（B_4O_7として）	0 － 2 %
カルボキシメチルセルロース	0 － 2 %
ポリマー（例えばPEG, PVP）	0 － 3 %
定着ポリマー例えば、ラウリルメタアクリレート／アクリル酸コポリマー；モノマー比25：1；MW 3800	0 － 3 %
グリセロール	0 － 5 %
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば分散剤、土壌抑制剤、香料、蛍光増白剤）	0 － 5 %

(7) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

脂肪アルコールスルファート	5－10%
エトキシル化脂肪酸モノエタノールアミド	3－9%
脂肪酸としての石けん	0－3%
炭酸ナトリウム（Na_2CO_3として）	5－10%
可溶性シリケート（Na_2O, $2SiO_2$として）	1－4%
ゼオライト（$NaAlSiO_4$として）	20－40%
硫酸ナトリウム（Na_2SO_4として）	2－8%
ナトリウムパーボラート（$NaBO_3 \cdot H_2O$ として）	12－18%
TAED	2－7%
ポリマー（例えばマレイン酸／アクリル酸コポリマー、PEG）	1－5%
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば螢光増白剤、土壌抑制剤、香料）	0－5%

(8) The detergent constituent blended as a granulated material which comes to contain the following components

直鎖アルキルベンゼンスルホナート（酸として計算）	8－14%
エトキシル化脂肪酸モノエタノールアミド	5－11%
脂肪酸としての石けん	0－3%
炭酸ナトリウム（Na_2CO_3として）	4－10%
可溶性シリケート（Na_2O, $2SiO_2$として）	1－4%
ゼオライト（$NaAlSiO_4$として）	30－50%
硫酸ナトリウム（Na_2SO_4として）	3－11%
クエン酸ナトリウム（$C_6H_5Na_3O_7$として）	5－12%
ポリマー（例えばPVP、マレイン酸／アクリル酸コポリマー、PEG）	1－5%
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば土壌抑制剤、香料）	0－5%

(9) The detergent constituent blended as a granulated material which comes to contain the following components

直鎖アルキルベンゼンスルホナート（酸として計算）	6－12%
非イオン界面活性剤	1－4%
脂肪酸としての石けん	2－6%
炭酸ナトリウム（Na_2CO_3として）	14－22%
ゼオライト（$NaAlSiO_4$として）	18－32%
硫酸ナトリウム（Na_2SO_4として）	5－20%
クエン酸ナトリウム（$C_6H_5Na_3O_7$として）	3－8%
ナトリウムパーボラート（$NaBO_3 \cdot H_2O$ として）	4－9%
漂白活性化剤（例えばNOBS又はTAED）	1－5%
カルボキシメチルセルロース	0－2%
ポリマー（例えばポリカルボキシレート又はPEG）	1－5%
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば蛍光増白剤、香料）	0－5%

(10) The aquosity liquid detergent constituent which comes to contain the following components

直鎖アルキルベンゼンスルホナート（酸として計算）	15－23%
アルコールエトキシスルファート（例えばC_{12-15}アルコール、2-3 EO）	8 －15%
アルコールエトキシラート（例えばC_{12-15}アルコール、7 EO、又はC_{12-15}アルコール、5 EO）	3 － 9 %
脂肪酸としての石けん（例えばラウリン酸）	0 － 3 %
アミノエタノール	1 － 5 %
クエン酸ナトリウム	5 －10%
ヒドロトロープ（例えばナトリウムトルエンスルホナート）	2 － 6 %
ボラート（B_4O_7として）	0 － 2 %
カルボキシメチルセルロース	0 － 1 %
エタノール	1 － 3 %
プロピレングリコール	2 － 5 %
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えばポリマー、分散剤、香料、蛍光増白剤）	0 － 5 %

(11) The aquosity liquid detergent constituent which comes to contain the following components

直鎖アルキルベンゼンスルホナート（酸として計算）	20－32％
アルコールエトキシラート（例えばC_{12-15}アルコール、7 EO、又はC_{12-15}アルコール、5 EO）	6 －12％
アミノエタノール	2 － 6 ％
クエン酸	8 －14％
ボラート（B_4O_7として）	1 － 3 ％
ポリマー（例えばマレイン酸／アクリル酸コポリマー、定着ポリマー、例えば、ラウリルメタクリレート／アクリル酸コポリマー）	0 － 3 ％
グリセロール	3 － 8 ％
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1％
少量成分（例えばヒドロトロープ、分散剤、香料、螢光増白剤）	0 － 5 ％

(12) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

アニオン界面活性剤（直鎖アルキルベンゼンスルホナート、アルキルスルファート、α－オレフィンスルホナート、α－スルホ脂肪酸メチルエステル、アルカンスルホナート、石けん）	25－40％
非イオン界面活性剤（例えばアルコールエトキシラート）	1 －10％
炭酸ナトリウム（Na_2CO_3として）	8 －25％
可溶性シリケート（Na_2O, $2SiO_2$として）	5 －15％
硫酸ナトリウム（Na_2SO_4として）	0 － 5 ％
ゼオライト（$NaAlSiO_4$として）	15－28％
ナトリウムパーボラート（$NaBO_3 \cdot 4H_2O$）として）	0 －20％
漂白活性化剤（TAED又はNOBS）	0 － 5 ％
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1％
少量成分（例えば香料、螢光増白剤）	0 － 3 ％

(13) It is the **** detergent compound indicated by 1-12, and all or a part of linear-alkyl-benzene sulfonate is permuted by alkyl (C12-C18) SURUFATO in here.
(14) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

（C_{12-18}）アルキルスルファート	9－15%
アルコールエトキシラート	3－6%
ポリヒドロキシアルキル脂肪酸アミド	1－5%
ゼオライト（$NaAlSiO_4$として）	10－20%
層状ジシリケート（例えばヘキスト社からのSK56）	10－20%
炭酸ナトリウム（Na_2CO_3として）	3－12%
可溶性シリケート（Na_2O, $2SiO_2$として）	0－6%
クエン酸ナトリウム	4－8%
ナトリウムパーカーボネート	13－22%
TAED	3－8%
ポリマー（例えばポリカルボキシラートおよびPVP）	0－5%
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば螢光増白剤、フォトブリーチ、香料、土壌抑制剤）	0－5%

(15) The detergent constituent blended as a granulated material which comes to contain the following components, and which has the bulk density of 600 g/l at least

（C_{12-18}）アルキルスルファート	4－8%
アルコールエトキシラート	11－15%
石けん	1－4%
ゼオライト MAP又はゼオライトA	35－45%
炭酸ナトリウム（Na_2CO_3として）	2－8%
可溶性シリケート（Na_2O, $2SiO_2$として）	0－4%
ナトリウムパーカーボネート	13－22%
TAED	1－8%
カルボキシメチルセルロース	0－3%
ポリマー（例えばポリカルボキシラートおよびPVP）	0－3%
酵素（純粋な酵素タンパク質として計算）	0.0001－0.1%
少量成分（例えば螢光増白剤、ホスホナート、香料）	0－3%

(16) The **** detergent constituent indicated by 1-15 containing the peroxy acid stabilized or enclosed as an alternative to the bleaching system which already made reference as an additional component.
(17) 1, 3, 7 with which par borate puts and is replaced by par carbonate
The **** detergent constituent indicated by 9 and 12.
(18) It is the detergent constituent indicated by 1, 3, 7, 9, 12, 14, and 15, and this contains a manganese catalyst additionally. A manganese catalyst may be one of the compounds indicated by "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, and pp.637-639.
(19) The detergent constituent blended as a non-aqueous detergent liquid which comes to contain a liquid nonionic surface active agent, for example, straight chain alkoxyl-ized primary alcohol, a builder system (for example, phosphate), an enzyme, and alkali. The detergent may contain the anionic surface active agent and/or the bleaching system again.
The lipase variant of this invention is blended by the concentration usually used in a detergent. Now, it sets to;, i.e., the detergent constituent of this invention, considered as follows, and the lipase variant of this invention is a lipase variant per 11. of washingses. It can add in the amount equivalent to 0.00001 to 1 mg (it calculates as pure enzyme protein).
Dishwashing constituent This surface active agent of a dishwashing detergent constituent may be an anion, non-ion, a cation, both sexes, or these types of mixture coming [a surface active agent]. Probably, the detergent contains 0-90% of nonionic surface active agent, for example, low foaming, thru/or the non-foaming ethoxyl-ized propoxyl-ized linear alcohol.
A detergent constituent can contain the detergent builder salt of inorganic and/or an organic type.
It is subdivided by the Lynn content type and the non-Lynn content type, and a detergent builder gets. A detergent constituent usually contains 1-90% of detergent builder.
When it exists, a water-soluble salt especially alkali-metal pyrophosphate, alt.phosphate, poly phosphate, and phosphonate are contained in the Lynn content inorganic alkali detergent builder's example. When it exists, it is the example of representation in which the various water-insoluble nature crystalline substances or amorphous aluminosilicates of a type were contained in the non-Lynn content inorganic builder's example at water-soluble alkali-metal carbo NATO, borate, and a silicate list, among these the zeolite was known most.
Alkali metal, ammonium and permutation ammonium, a citrate, succinate, malonato, a fatty-acid sulfonate, carboxymethoxy succinate, ammonium polyacetate, carboxylate, poly carboxylate, amino poly carboxylate, poly acetyl carboxylate, and a polyhydroxy sulfonate are contained in a suitable organic builder's example.
The amount polymer of macromolecules and copolymer by which having a builder property is known, for example, suitable polyacrylic acid, polymer lane acids / polyacrylic acid copolymers, and those salts are contained in other suitable organic builders.
A dishwashing detergent constituent can contain a bleaching agent chlorine / bromine type, or oxygen type. The example of a bleaching agent inorganic chlorine / bromine type is chlorination TORINA thorium phosphate at a lithium, sodium or a calcium hypo chlorite, and a high POBURO dynamite list. The examples of a bleaching agent organic chlorine / bromine type are heterocycle type N-BUROMO and N-chloro

imide, for example, TORIKURORO isocyanuric acid, TORIBUROMO isocyanuric acid, dibromo isocyanuric acid, dichloro isocyanuric acid, a water solubilization cation, for example, a potassium, and sodium, and those salts. A hydantoin compound is also suitable.
an enzyme bleaching agent -- for example, the form of pel salt (persalt) -- it is -- desirable -- a bleaching agent precursor -- or it is desirable as a peroxy acid compound. The typical examples of a suitable peroxy bleaching agent compound are alkali-metal par borate, both the tetrapod hydrate and a mono-hydrate, alkali-metal par carbo NATO, par silicate, and par phosphate. Desirable activator matter is TAED and glycerol triacetate.
The dishwashing detergent constituent of this invention can be stabilized using a stabilizer, for example, polyol, for example, propylene glycol, sugar or sugar-alcohol, a lactic acid, a boric acid, or a boric-acid derivative, for example, aromatic series borate ester, usual [to an enzyme (1 or more)].
A dishwashing detergent constituent can contain other enzymes especially amylases, proteases, and/or cellulases again.
The dishwashing detergent constituent of this invention may also contain other detergent component, for example, deflocculatant matter, bulking agent matter, defoaming agent, anticorrosive agent, soil suspending agent, sequestering agent, anti-soil reattachment agent, dehydrating agent, color, germicides, fluorescence agents, usual thickeners, and usual perfume again.
At the end In one of the dishwashing detergents usual in the variant of this invention, for example, a detergent given in either of the following patent official reports : which can be used -- EP 551670 -- EP 533239, WO 9303129, and EP 507404, US5141664, GB2247025 and EP414285, GB 2234980, EP 408278, GB 2228945, GB 2228944, EP 387063, EP 385521, and EP 373851, EP 364260, and EP 349314, EP 331370, and EP 318279, EP 318204, and GB 2204319, EP 266904, and US 5213706, EP530870, CA2006687 and EP481547, EP 337760, WO 93/14183, US 5223179, WO 93/06202, WO 93/05132, WO 92/19707, and WO 92/09680, WO 92/08777, WO 92/06161, WO 92/06157, WO 92/06156, WO 91/13959, EP 399752, US 4941988, US 4908148.
a lipase variant -- for example, -- Surfactant and Consumer Products edited by J.Falbe, 1987, pp 295-296;Tenside Surfactants Detergents, and 30 (1993) -- 6, pp 394-399;JAOCS, Vol.61 (1984), 2 and pp 367-376;EP 517 762;EP 123 400;WO 92/19714;WO 93/19147;US 5,082,578;EP 494 769;EP 544 493;EP 543 562;US 5,235,082;EP It can be used in the **** textile softening agent indicated by 568 297;EP 570 237.
This invention is further explained in an attached drawing.
Drawing 1 is the restriction map of pYESHL, drawing 2 is the restriction map of a plasmid pAO1, and drawing 3 is the restriction map of Plasmid pAHL, and drawing 4 and drawing 5 are construction of the gene which carries out the code of the variant of this invention.
Although the following example explains this invention further, in any cases, the range of this invention is not restricted.
The matter and approach DOITCHIE ZAMMURUGU Phone Micro ORUGA varnish noodle UNTO TSUERUKARUTSUREN GmbH, MASUSHIRODERUBEKU1b, D-330 Brunswick, Fumi Kola available from the German republic RANUGINOSA (Humicola lanuginosa) DSM 4109.
pYESHL(s) are yeast / E. collie shuttle vector lipase, it is discovered in yeast and this secretes H. RANUGINOSA lipase of a low in yeast. More specifically, pYESHL is the derivative of pYES(purchased from in vitro gene company and UK) 2, GAL1

promotor is excised in here, and they are a Fumi Kola RANUGINOSA lipase gene and TPI (triose phosphate isomerase) (Alvar, T. and KAWASAKI, G., and J.Mol.Appl.Genet 1,419-434 (1982) were cloned between SphI and a XbaI part.) from S. SEREBISHIE. The restriction map of pYESHL is shown in drawing 1 .
The first protein joint filter (nylon film) and the second low protein joint filter (cellulose acetate) are prepared in a low calcium filter assay procedure 1SC Ura replica plate (useful to selection of the strain which has an expression vector) at a crowning.
2) Open the yeast which contains a parent lipase gene or a mutation lipase gene on a duplex filter, and incubate for two days or three days at 30 degrees C.
3) Hold a colony on a top filter by moving a top filter to a new plate.
4) In order to empty a Petri dish, remove a protein joint filter.
5) In order to identify the colony which discovers lipase activity in the form of a blue-green spot, they are an olive-oil emulsion (2%P.V.A.: olive-oil =3:1), the brilliant green (an indicator, 0.004%), 100 non-tris-buffers pH9, and EGTA (last concentration 5mM).
The agarose solution which becomes by ****** is poured out on a bottom filter.
6) Identify the colony found out at the process 5 which has the dependency by which calcium was made to decrease as compared with parent lipase.
Dobanol (trademark) 25-7 filter assay: Screening for the resistance improved to a detergent component is performed by use of the filter assay which is equivalent to above it except for the fact that the solution which 5 defined contains Dobanol (trademark) 25-7 0.02 more%.
Use of the construction a all lipase code gene of a random mutation-ized library
Plasmid pYESHL is processed at a room temperature for 20 minutes using 12M formic acid. A generation lipase code gene is increased from a formic-acid processing plasmid using the conditions (1989 reference, such as 0.5mM MnCl2 and ATP of the normal amount of 1/5, for example, Leung etc.) of mutation.
;, because the formic acid with which it is expected that this processing will give wide range mutation -- mainly -- transformer bar SHON -- giving -- and -- The mutation completed by PCR mainly gives a rearrangement.
It was obtained. An PCR fragment is cloned by the transformation of digestion into duplex recombination (1992, such as Muhlrad) or a shuttle vector, association, and E. collie into a shuttle vector by in vivo one.
The array arrangement of the clone picked up to eight random was carried out, and having an average of two to three mutation on the both sides of transformer bar SHON and a rearrangement was found out.
By this approach, seven sorts of libraries are 10,000-140,000. It was created so that a clone might be contained.
b) Operation of the localization random mutagenesis A mutation primer (oligonucleotide) is compounded and this should be mutation-ized except for the nucleotide (1 or more) equivalent to the amino acid codon (1 or more) which should be mutation-ized. It is equivalent to a part of DNA array.
Then, it is a suitable opposite primer about the obtained mutation primer. It uses at an PCR reaction. It was obtained. An PCR fragment is refined, and subsequently it digests and, subsequently to in a shuttle vector, clones. If alternatively and required, it generates. It is the second with the second suitable opposite primer as a primer so that an PCR fragment may be made to digest and cloning of a mutation-ized field may be made to make in a shuttle vector. It is used at an PCR reaction. An PCR reaction is performed under the usual conditions.

DNA sequencing follows the inside of an ABI die terminator cycle sequencing kit, and a protocol, and is Applied Biosystem. It carried out using ABI DNA array model 373A.
example 1 of an example Construction of a random lipase variant all H. RANUGINOSA lipase genes and amino acid (aa)91-97 of those -- and -- 206-211 The random mutation-ized library was created as indicated by said matter and approach. It reaches amino acid field 91-97. 206-211; selected to the first round of the localization mutagenesis, because these fields are because the important thing was found out to the washing engine performance. A field 91-97 is a part of lid (lid) field of lipase, and is a field. 206-211 A part of hydrophobic KUREFUTO (creft) of lipase is constituted.
One oligonucleotide was compounded to each of these fields that come to contain each 2.33% of other three nucleotides by the amino acid codon expected 93% of wild type nucleotide, and mutation-ization. There was no change of amino acid, and when possible, bigger possibility was acquired to the change to the amino acid which compounds the third nucleotide (Wobble base) in a codon by C G/50% 50%, and has one piece or two codons.
The presentation of the oligonucleotide of the mutation of a field 91-97 is shown in Table 1.
By use of this oligonucleotide, the mutation frequency by which about 65 to 70% was calculated was obtained in the library to one amino acid change introduced into parent lipase. The frequency of the mutation to introduced two pieces or the amino acid change beyond it is less than 35%. It is brought that this low mutation frequency is chosen and the observed amino acid change in an electropositive colony improves an enzyme, and it secures that it is not a "neutral" (neutral) change exactly because of high mutation frequency.
About a mutation primer, it is a suitable opposite primer. It used at the PCR reaction. It was obtained. An PCR fragment is refined and it is a field. 206-211 It was digested at the case and cloned in the shuttle vector. In the case of the field 91-97, it was obtained. It is the second with the second suitable opposite primer, using an PCR fragment as a primer. It used at the PCR reaction. This process was digested, and it was required in order to clone a mutation-ized field in a shuttle vector.
The library of a field 91-97 and a field 206-211 was created, and this contained the 10,000 - 80,000 clone / library. Most colonies are positivities (90% or more), when inspecting under conditions in case parent lipase is a positivity, namely, they show lipase activity. The positive reaction was measured by filter assay about 2.5mM calcium (to substitute of 5mM EGTA).
From a different library using the low calcium assay indicated by Dobanol (trademark) 25-7, the aforementioned matter, and the approach 450,000 colonies were screened. aa 25 low calcium positivity objects from a 91-97 library C lid-field and 12 Dobanol(trademark)25-7 positivity objects from all gene libraries were isolated. aa Sequencing of the 14 low calcium positivity objects was carried out from the mutagenesis of 91-97.
Other three mutation of the outside of a mutation-ized field (it can set to a codon 83,103,145)
Mutation which is S83T although ** and PCR incorrect incorporation can explain It is unusual transformer bar SHON to PCR incorrect incorporation.

配列：

5′	5	C	G
T	5	C	3′
T	7	A	
A	8	G	ボトル 5：93% A; 2.33% C; 2.33% G and 2.33% T
T	8	T	
T	A/C	T	
T	5	C	
C	7	T	
T	5	C	ボトル 6：93% C; 2.33% A; 2.33% G and 2.33% T
T	8	T	
T	8	A	
6	C/G	T	
5	6	G	ボトル 7：93% G; 2.33% A; 2.33% C and 2.33% T
5	6	G	
7	G	A	
8	A	A	
6	T	C	ボトル 8：93% T; 2.33% A; 2.33% C and 2.33% G
7			

表１：リボラーゼ（商標）のアミノ酸91－97の局在ランダム変異誘発に対し用いられるオリゴヌクレオチドの構築の説明。配列中に存在する番号は、ボトルを意味し、そのボトルの組成は配列の各側に現われる。

表　2

菌株番号	変異体タイプ						
59	I			G91A	N94K		D96A
60	II	S83T			N94K		D96N
61	II	S83T			N94K		D96N
62	III		E87K				D96V
63	IV		E87K	G91A			D96V
64	II	S83T			N94K		D96N
65	III		E87K				D96V
67	V				N94K	F95L	D96H
69	V				N94K	F95L	D96H
71	III		E87K				D96V
72	II	S83T			N94K		D96N

表２：菌株番号は、アスペルギルス発現ベクターPAHL内にクローン化される当初に選ばれたクローンを言及する。変異体タイプは、同じクローンを言及しておりこのクローンは恐らくランダム突然変異化ライブラリーの増幅中に生じたものであろう。変異体タイプⅠおよびⅡは、0.01% Dobanol（商標）25－７において活性であり、一方残りは野生型に似て不活性である。

表　3

菌株番号	変異体タイプ	DNA配列（配列の上のアミノ酸番号）										
		82	83	84	85	86	87	88	89	90	91	92
wt		GGC	TCT	CGT	TCC	ATA	GAG	AAC	TGG	ATC	GGG	AAT
59	I										C	
60	II		A								C	
61	II		A								C	
62	III						A				C	
63	IV						A				C	
64	II		A								C	
65	III						A				C	
67	V										C	
52/68	wt											
53	wt											
69	V										C	
71	III						A				C	
72	II		A								C	
73	VI											

菌株番号	変異体タイプ	93	94	95	96	97	98	99	100	-103	-145
wt		CTT	AAC	TTC	GAC	TTG	AAA	GAA	ATA	-ATT	-CAT
59	I	G	G		C						
60	II	G	G		A						
61	II	G	G		A						
62	III				T						
63	IV				C					C	C
64	II	G	G		A						
65	III	G			T						
67	V		A	C	A	C					
52/68	wt										
53	wt										
69	V		A	C	A	C					
71	III	G			T						
72	II	G	A		A						
73	VI				A	?					

表３：野生型配列は最上ラインに示される。wtとは異なるヌクレオチドのみが、変異体配列に示される。コドン91および93の塩基を、それぞれＣ／ＴおよびＴ／Ｇの１：１でドープした。別に、コドン91－97でのヌクレオチドを93％wtおよび2.33％の３種の他のヌクレオチドを用いてドープした。

Example [ ] 2 by the approach indicated in Example 1, and the same approach, the following variant was built by the random mutagenesis. The actual screening criteria used in order to choose some variants are also shown.
D167 G+E210V5mM EGTA and 0.01% Dobanol (trademark) 25-7 and 0.006% LASE87 K+G91 A+L93 I+N94 K+D96 A5mM EGTA, 0.02% Dobanol(trademark)25-7N73 D+S85 T+E87 K+G91 A+N94 K+D96AS83 T+E87 K+W89 G+G91 A+N94 K+D96VE87 K+G91 A+D96 R+I100VS83 T+E87 K+Q249RE87 K+G91A example 3 Aspergillus Fumi Kola in the inside of ORIZE

Manifestation of RANUGINOSA lipase Fumi Kola Cloning of RANUGINOSA lipase is indicated by EP305216. Moreover, EP305216 is an Aspergillus. A manifestation and the description of the lipase in the inside of ORIZE are indicated. The used manifestation plasmid is named p960.
The manifestation plasmid used with this application is immediately the same as that of p960 except for slight qualification of 3' to a lipase coding region. Qualification digested :p960 performed as follows with NruI and a BamHI restriction enzyme. Between these two parts, the BamHI/NheI fragment (the fill-in of the NheI fragment was carried out by the Klenow polymerase here) from a plasmid pBR322 is cloned, this creates a plasmid pA01 (drawing 2), and this contains peculiar BamHI and a NheI part. These peculiar part BamHI/XbaI fragments from p960 were cloned, and pAHL (drawing 3) was obtained.
site specific in vitro mutagenesis of a lipase gene the approach used in order to introduce mutation into a lipase gene -- Nelson and -- It is indicated by long, Analytical Biochemistry, and 180,147-151 (1989). This approach was chemically compounded as one of the primers in the PCR reaction. 3 process creation of PCR (polymerase chain reaction) containing mutation to have been introduced using the DNA strand is included. From an PCR creation fragment, it has variation. A restriction enzyme can decompose and a DNA fragment can carry out reinsertion to an expression vector. This approach is completely indicated by Example 5. In drawing 4 and drawing 5 , this approach is outlined further.
FUMIKORA Construction of the plasmid which discovers the N94 K/D96A analog of RANUGINOSA lipase the line of Plasmid pAHL ---izing the circular plasmid pAHL -- reaction mixture:50mM NaCl of the following 50microl, and 10mM tris - the inside of MgCl2 of HCl, pH 7.9, and 10mM, the dithiothreitol of 1mM, the plasmid of 1microg, and SpHI of two units, and restriction enzyme It line-izes using SphI. It digests at 37 degrees C for 2 hours. A phenol (they are equilibration and pH7.5 at tris-HCl) extracts a reaction mixture, subsequently, it adds and 96% ethanol of ice-cooling of 2 capacity is settled. Centrifugal Perilla frutescens (L.) Britton var. crispa (Thunb.) Decne. was carried out, and it line-ized after drying a pellet. DNA was dissolved in the water of 50microl and, subsequently concentration was presumed on agarose gel.
Three processes PCR mutagenesis As shown in drawing 3 , 3 process mutagenesis includes use of four sorts of primers. :

変異誘発プライマー(＝A) : 5'-TATTTCTTTCAAAGCGAACTTAAGATTC-CCGAT-3'

PCR ヘルパー1 (＝B) : 5'-GGTCATCCAGTCACTGAGACCCTCTACCTATTAA-ATCGGC-3'

PCR ヘルパー2 (＝C) : 5'-CCATGGCTTTCACGGTGTCT-3'

PCR ハンドル (＝D) : 5'-GGTCATCCAGTCACTGAGAC-3'

With the array of the outside of a coding region, the helper 1 and the helper 2 are complementary, and can follow, and can use combining a mutagenesis primer in construction of a variant array. All three processes are :10mM tris performed in the following buffer solution containing the following components. - They are HCl, pH 8.3, 50mM KCl, 1.5mM MgCl2, and 0.001%. Gelatin, 0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.2mM TTP, 2.5 units Taq polymerase.
a process 1 -- setting -- 100pmol Primer A and 100pmol Primer B and 1fmol -- a line -

- a-izing plasmid is added to the reaction mixture of a total of 100microl, and 15 times of the cycles which consist at 95 degrees C for 2 minutes, and consist of 3 minutes at 2 minutes and 72 degrees C by 37 degrees C are performed.
The concentration of an PCR product is presumed by agarose gel. Subsequently, a process 2 is performed. 0. 6pmol process 1 product and 1fmol -- a line -- a-izing plasmid performs 1 cycle of which it consists at 95 degrees C for 5 minutes, and it consists from 10 minutes at 2 minutes and 72 degrees C by 37 degrees C by being contained in said buffer solution of 100microl.
the reaction mixture of a process 2 -- 100pmol Primer C and 100pmol(s) Primer D is added (each 1microl) and 20 times of the cycles which consist at 95 degrees C for 2 minutes, and consist of 3 minutes at 2 minutes and 72 degrees C by 37 degrees C are performed. This actuation included the process 3 in the mutagenesis procedure.
Isolation of a mutation restriction fragment The product of a process 3 is isolated from agarose gel, and, subsequently to the inside of H2O of 20microl, is remelted. Subsequently, :100mM NaCl, 50mM tris which are digested using restriction enzymes BamHI and BstXI in the total capacity l of 50micro which has the following presentations - MgCl2 of HCl, pH 7.9, and 10mM, 1mM DTT, BamHI of ten units, and BstXI of ten units. An incubation is performed at 37 degrees C for 2 hours. A 733 bp BamHI/BstXI fragment is isolated from agarose gel.
Ligation to expression vector pAHL The manifestation plasmid pAHL is cut using the bottoms BamHI and BstXI of a condition of the above, and this big fragment is isolated from agarose gel. The mutation fragment isolated previously is combined with this vector, and a transformation is carried out to E. collie using joint mixture. Cutting and orientation of a fragment are proved by cutting of the plasmid preparation from the transformant by the restriction enzyme. Analysis of an array is ABI DNA. It carries out about a double strand plasmid using the DyeDeoxy (trademark) terminator cycle sequencing kit on a sequencer (Applied Biosystem). The plasmid is the same as that of pAHL except for the codon which was named pAHLG91 A/N94 K/D96A and was permuted.
Example [ ] 4 Fumi Kola Construction of the plasmid which discovers other variants of lipase The following variant is built using the approach which ******(ed) example 3, and the same approach. **** and the primer of a plasmid which were used to qualification are hung up over below.

プラスミド命称	プライマーA配列
pAHLS83T/N94K/D96A	5′-ATTTCTTTCAAAGCGAACTTAAGATTCCCGA-TCCAGTTCTCTATGGAACGAGTGCCACGGAAAGA-3'
pAHLE87K/D96V	5-TATTTCTTTCAAAACGAAGTTAAGATTCCCGATCC-AGTTCTTTATGGAACGAGA-3'
pAHLE87K/G91A/D96A	5′-TATTTCTTTCAAAGCGAAGTTAAGATTAGCGATC-CAGTTCTTTATGGAACGAGA-3'
pAHLN94K/F95L/D96H	5'-TATTTCTTTCAAGTGCAACTTAAGATTCCCGAT-3'
pAHLF95C/D96N	5'-TATTTCTTTCAAGTTACAGTTAAGATTCCC-3'
pAHLG91S/L93V/F95C	5′-TATTTCTTTCAAGTCACAGTTAACATTAGAGATCC-AGTTCTC-3'

pAHLE87K/G91A/L93I/N94K/D96A

5'-TATTTCTTTCAAAGCGAACTTAATATTAGCGATC-CAGTTCTTTATGGAACGAGA-3'

pAHLD167G 5'-ATATGAAAACACACCGATATCATACCC-3'

pAHLA121V 5'-CCTTAACGTATCAACTACAGACCTCCA-3'

pAHLR205K/E210Q 5'-GCTGTAACCGAATTGGCGCGGCGGGAGCTTAGGG-ACAATATC-3'

pAHLN73D/S85T/E87K/G91A/N94K/D96A

5'-TATTTCTTTCAAAGCGAACTTAAGATTAGCGATC-CAGTTCTTTATAGTACGAGAGCCACGGAA-AGAGAGGACGATCAATTTGTCCGTGTTGTCGAG-3'

pAHLS83T/E87K/W89G/G91A/N94K/D96V

5'-TATTTCTTTCAAAACGAACTTAAGATTAGCGATA-CCGTTCTTTATGGAACGAGTGCCACGGAAAGA-3'

pAHLE87K/G91A/D96R/I100V

5'-GCAAATGTCATTAACTTCTTTCAATCTGAAGTTAA-GATTAGCGATCCAGTTCTTTATGGAACGAGA-3'

pAHLS83T/E87K 5'-CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-AAAGA-3'

pAHLE87K/G91A 5'-GAAGTTAAGATTAGCGATCCAGTTCTTTATGGAA-CGAGA-3'

pAHLS83T/E87K 5'-CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-AAAGA-3'

pAHLQ249R 5'-CGGAATGTTAGGTCTGTTATTGCCGCC-3'

Example [ ] 5 Fumi Kola Construction of the plasmid which discovers the combination analog of lipase Plasmid pAHLD167 G/E210V pAHLA121V/R205K/E210Q and -- pAHLS83 T/E87 K/Q249R is built by performing two continuous mutagenesis processes using a suitable primer.

Example [ ] 6 manifestation of the lipase analog in the inside of an Aspergillus

Aspergillus Transformation (general procedure) of ORIZE

100ml YPD (Methods in Yeast Genetics, such as Sherman, Cold Spring Harbor Laboratory, 1981) was inoculated by the spore of A. ORIZE, and subsequently, it shook for about 24 hours and incubated. It collects by filtering a hypha through Myra Klos (miracloth), and is 200ml. It washes by MgSO4 of 0.6M. It is 15ml about a hypha. 1.2M MgSO4, 10mM It washes by NaH2PO4 (pH=5.8). Suspension is cooled in Hikami and, subsequently it is 120mg. The 1ml buffer solution containing NOZAZAIMU (trademark) and a batch 1687 is added. until it is visible in the sample which adds 12mg/1ml ml BSA (sigma type H25) and by which many protoplasts are subsequently observed under a microscope after 5 minutes -- 37 degrees C 1.5-2.5 time amount -- the incubation accompanied by quiet churning was continued. Suspension is filtered through Myra Klos, filtrate is moved to sterilization tubing, and it is 5ml. 0.6M sorbitol and 100mM Tris - It **** by HCl (pH=7.0). Centrifugal is

performed for 15 minutes by 1000g, and protoplasts are collected from the crowning of MgSO4 cushion. STC (1.2M sorbitol, 10mM tris-HCl, pH=7.5, 10mM CaCl2) of 2 capacity is added to protoplast suspension, and, subsequently the at-long-intervals alignment of the mixture is carried out by 1000g for 5 minutes. It is 3ml about protoplast PURETTO. It is made to make re-[in STC] suspend and re-pelletize. It is ********** about this. To the last, it is 0.2 to 1 ml about a protoplast. It is made to re-suspend in STC.

About the protoplast suspension of 100microl, they are p3SR2 (A. NIDORA lance amdS gene which has the plasmid indicated by 1983 in Mol.and Cel.Biol. [, such as Hynes,], Vol.3, and No.8 1430 -1439 or August) of 5-25microg, and 10microl. It mixes in STC. Mixture is left for 25 minutes at a room temperature. 0.2ml 60% PEG4000 (BDH29576) and 10mM Tris of CaCl2 and 10mM(s) - HCl (pH=7.5) is added, it mixes carefully, finally this 0.85ml solution is added, and it mixes carefully. Mixture is left for 25 minutes at a room temperature, and it rotates for 15 minutes by 2,500g, and is 2ml about a pellet. It is made to re-suspend in 1.2M sorbitol. Once again, after sedimentation, a protoplast is sprinkled on the minute play containing the acetamide and 20mM Cscce of 10mM(s) as 1.0M sucrose, pH=7.0, and a nitrogen source, and background (hackground) **** is prevented.

Gather and collect spores after an incubation for four - seven days at 37 degrees C, and sterilization underwater is made to suspend, and it extends for a single colony. This procedure is ********(ed) and it saves as a transformant which defined the kind of the second single colony after re-isolation.

A. Manifestation of the lipase homolog in the inside of ORIZE As said example indicated said plasmid, the amdS gene from A. nidulans is contained. A transformation is carried out into A. ORIZE IFO 4177 by the joint transformation using p3SR2.

About the prepared protoplast, it is a manifestation plasmid like the above. The equivalent mixture of p3SR2 is incubated and about 5microg is used respectively. The transformant which used the acetamide as an only nitrogen source was re-isolated twice. For three days The supernatant of a culture is analyzed after growth using the analysis for lipase activity on YPD. a further research of the transformant of good quantity sake -- choosing -- and -- FG4 200ml culture medium (an soybean mill, 3% malto dextrin, 1% peptone, and pH are adjusted to 7 by 4M NaOH 3%) -- the upper -- it increases at 30 degrees C for four days by 30 degrees C within a 1l. shaking flask. Purification of the lipase variant of example 7 this invention Analysis of lipase activity: The substrate to lipase was manufactured by making glycine TORIBUCHIRATO (Merck) emulsify using gum arabic as an emulsifier.

Lipase activity was analyzed by pH7 using the pH stat method. One unit of lipase activity (LU/mg) was defined as a complement, in order to separate the fatty acid of per minute 1 micromole.

Process 1: Carry out centrifugal [of the fermentation supernatant] and it is ****** about precipitate. pH of a supernatant is adjusted to 7 and equivalent 96% ethanol of colds is added gradually. Mixture is left for 30 minutes in an ice bath. Centrifugal Perilla frutescens (L.) Britton var. crispa (Thunb.) Decne. is carried out, and it is ****** about precipitate.

Process 2: Ion exchange chromatography. It applies to the DEAE-high-speed style (Pharmacia (trademark)) column which filtered the supernatant and was equilibrated with the 50mM tris-acetate buffer solution (pH=7). A column is washed with this buffer solution until absorption by 280nm is set to 0.05 or less OD. the five capacity of column -- using -- the line of the same buffer solution -- the enzyme activity

combined by salt gray JIENTO (NaCl of 0-5M) is made to flow out The fractionation containing enzyme activity is collected.
Process 3: Hydrophobic chromatography. The mol concentration of the pool which has enzyme activity is adjusted to 0.8M by adding solid-state ammonium acetate. Enzyme TSK gel butyl-toe yaw pearl It is this column although applied on a 650C column (available from Toso and Japan). Preliminary equilibration was carried out with 0.8M ammonium acetate. The uncombined matter is washed with 0.8M ammonium acetate, and elution of the cementing material is carried out with distilled water.
Process 4: Dilute with water the pool which has lipase activity, and adjust it to conductance 2mS and pH7. A pool is applied to the high performance Q sepharose (FARU bowfin) column which carried out preliminary equilibration with the TORISU acetate buffer solution (pH7) of 50mM(s). The united enzyme is made to flow out by straight-line salt gray JIENTO.
Example [ ] 8 washing engine performance of the lipase variant of this invention Fumi Kola of this invention As compared with wild type H. RANUGINOSA lipase, ** was in OD280, and the washing capacity of RANUGINOSA (Humicola lanuginosa) lipase was evaluated on the basis of the enzyme dosage of mg unit of the protein perl.
The washing trial was placed into aqueous [by which a temperature setup was carried out]. It carried out in the 150ml beaker. The beaker was agitated with the triangular MAG rod.
The experiment conditions were as follows.
approach: -- 3 times cycle washings: accompanied by overnight desiccation to between each cycle -- per beaker 100mlSUWATCHI: -- six SUWATCHI (3.5x3.5cm) textile per beaker: -- 100% cotton and lard which soaked trial textile style #400 and was colored in :Sudan red (0.75mg [per lard 1g] color). The lard of 6microl heated at 70 degrees C was applied to the core of each SUWATCHI. SUWATCHI was heated in ** during 30 minutes at 75 degrees C after applying a stain. Subsequently, overnight preservation of SUWATCHI was carried out at the room temperature before the first washing.
Detergent : LAS (NANSA1169/p, 30% a.m.) 1.17 g/l AEO (Dobanol 25-7 (trademark)) 0.15 g/l Sodium Triphosphate 1.25 g/l Sodium SURUFATO 1.00 g/l Sodium Carbo NATO 0.45 g/l Sodium Silicate 0.15 g/lpH:10.2 lipase concentration: -- per [0.075, 0.188, 0.375, and 0.75]l. and lipase protein 2.5mg time amount: -- 20-minute concentration: -- 30 degrees C -- rinsing -- :tap water -- a 15 minute desiccation:room temperature -- night (- 20 degrees C and 30 - 50% RH)
Evaluation: The reflection factor in 460nm was measured after 3 times washing.
Result The dose-response curve was compared with a lipase variant and natural H. RANUGINOSA lipase. It calculated by fitting the data measured in the dose-response curve to the following equality. :

$$\Delta R = \Delta R_{max} \frac{C^{0.5}}{K + C^{0.5}} \qquad (I)$$

deltaR is effectiveness expressed in reflection factor here, and C is enzyme concentration (mg/ml).
It comes out, and it is, and deltaRmax is a constant showing optimum, K is a constant, and;K2 express the enzyme concentration from which the one half of optimum is obtained.
The improvement factor was calculated based on characteristic constant deltaRmax and K which were found out by wild type lipase at each lipase variant list. Degree

type II f improvement = CWT/C (II)
The improvement factor which defined by carrying out expresses the amount of the lipase protein needed for acquiring the same effectiveness as the effectiveness acquired about the contrast wild type protein (CWT) of 0.25 mg/l.
Therefore, the effectiveness (deltaRwild-type) of the wild type protein in :1 0.25 mg/l whose procedure which calculates an improvement factor was as following was calculated using equality (I).;
2) Concentration of the lipase variant which brings about the same effectiveness as a wild type by 0.25 mg/l.

$$C = \left(K_{(類似体)} \frac{\Delta R_{(野生型)}}{\Delta R_{max(類似体)} - \Delta R_{(野生型)}}\right)^2 \qquad (III)$$

3) The improvement factor was calculated by equality (II). A result is shown in the following table 1.

表　1

变异体	改善因子
E87K+D96V	1.2
S83T+N94K+D96N	2.3
N94K+D96A	2.7
E87K+G91A+D96A	2.6
N94K+F95L+D96H	3.3
D167G+E210V	5.0
E87K+G91A+L93I+N94K+D96A	1.3
E87K+G91A+D96R+I100V	5.2
E87K+G91A	5.0
N73D+E87K+G91A+N94I+D96G	1.3
S83T+E87K+G91A+N92H+N94K+D96M	3.8
K46R+E56R+G61S	1.9
D102K	0.2
D167G	1
N73D+E87K+G91A+N94I+D96G	1.3
E210R	2.7
E210K	5.5
E210W	1
N251W+D254W+T267W	0.8
S83T+E87K+G91A+N92H+N94K+D96M	3.8
E56R+I90F+D96L+E99K	4.8
D57G+N94K+D96L+L97M	1.9

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（C）鎖の数：一本鎖

（D）トポロジー：直鎖状

（ii）配列の種類：cDNA

（vi）起源：

（A）生物名：フミコラ　ラヌギノサ

（ix）配列の特徴

（A）名称／キー：CDS

（B）位置：1..873

（C）名称／キー：　mat－ペプチド

（D）位置：67..83

（xi）配列：配列番号：1：

```
ATG AGG AGC TCC CTT GTG CTG TTC TTT GTC TCT GCG TGG ACG GCC TTG   48
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
       -20                 -15                 -10

GCC AGT CCT ATT CGT CGA GAG GTC TCG CAG GAT CTG TTT AAC CAG TTC   96
Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
    -5                  1                   5                  10

AAT CTC TTT GCA CAG TAT TCT GCA GCC GCA TAC TGC GGA AAA AAC AAT  144
Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
                15                  20                  25

GAT GCC CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC  192
Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
            30                  35                  40

GAG GTA GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT  240
Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
        45                  50                  55

GGA GTG GGC GAT GTC ACC GGC TTC CTT GCT CTC GAC AAC ACG AAC AAA  288
Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
    60                  65                  70

TTG ATC GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAG AAC TGG ATC  336
Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
75                  80                  85                  90

GGG AAT CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC  384
Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
                95                  100                 105
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TGC AGG GGA CAT GAC GGC TTC ACT TCG TCC TGG AGG TCT GTA GCC GAT  432
Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
            110                 115                 120
ACG TTA AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT  480
Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
        125                 130                 135

CGC GTG GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT  528
Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
    140                 145                 150

GCC GGA GCA GAC CTG CGT GGA AAT GGG TAT GAT ATC GAC GTG TTT TCA  576
Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155                 160                 165                 170

TAT GGC GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC  624
Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
                175                 180                 185

GTA CAG ACC GGC GGA ACA CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT  672
Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
            190                 195                 200

GTC CCT AGA CTC CCG CCG CGC GAA TTC GGT TAC AGC CAT TCT AGC CCA  720
Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
        205                 210                 215

GAG TAC TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAT  768
Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
    220                 225                 230

ATC GTG AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT  816
Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

AAC ATT CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG  864
Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
                255                 260                 265

ACA TGT CTT TAGTGGCCGG CGCGGCTGGG TCCGACTCTA GCGAGCTCGA GATCT    918
Thr Cys Leu
```

（２）配列番号：２に対する情報

（ｉ）配列の特徴

（Ａ）長さ：　291個のアミノ酸

（Ｂ）タイプ：アミノ酸

（Ｄ）トポロジー：直鎖状

（ii）配列の種類：タンパク質

（xi）配列：配列番号：２：

```
Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu
      -20                 -15                 -10

Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe
   -5                  1                   5                  10

Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn
                15                  20                  25

Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro
            30                  35                  40

Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser
        45                  50                  55

Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys
    60                  65                  70

Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile
75                  80                  85                  90

Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly
                95                  100                 105

Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp
            110                 115                 120

Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr
        125                 130                 135

Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val
    140                 145                 150

Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser
155                 160                 165                 170

Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr
                175                 180                 185

Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile
            190                 195                 200

Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro
        205                 210                 215

Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp
    220                 225                 230

Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro
235                 240                 245                 250

Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly
                255                 260                 265

Thr Cys Leu
```

[Translation done.]

DRAWINGS

[Drawing 1]

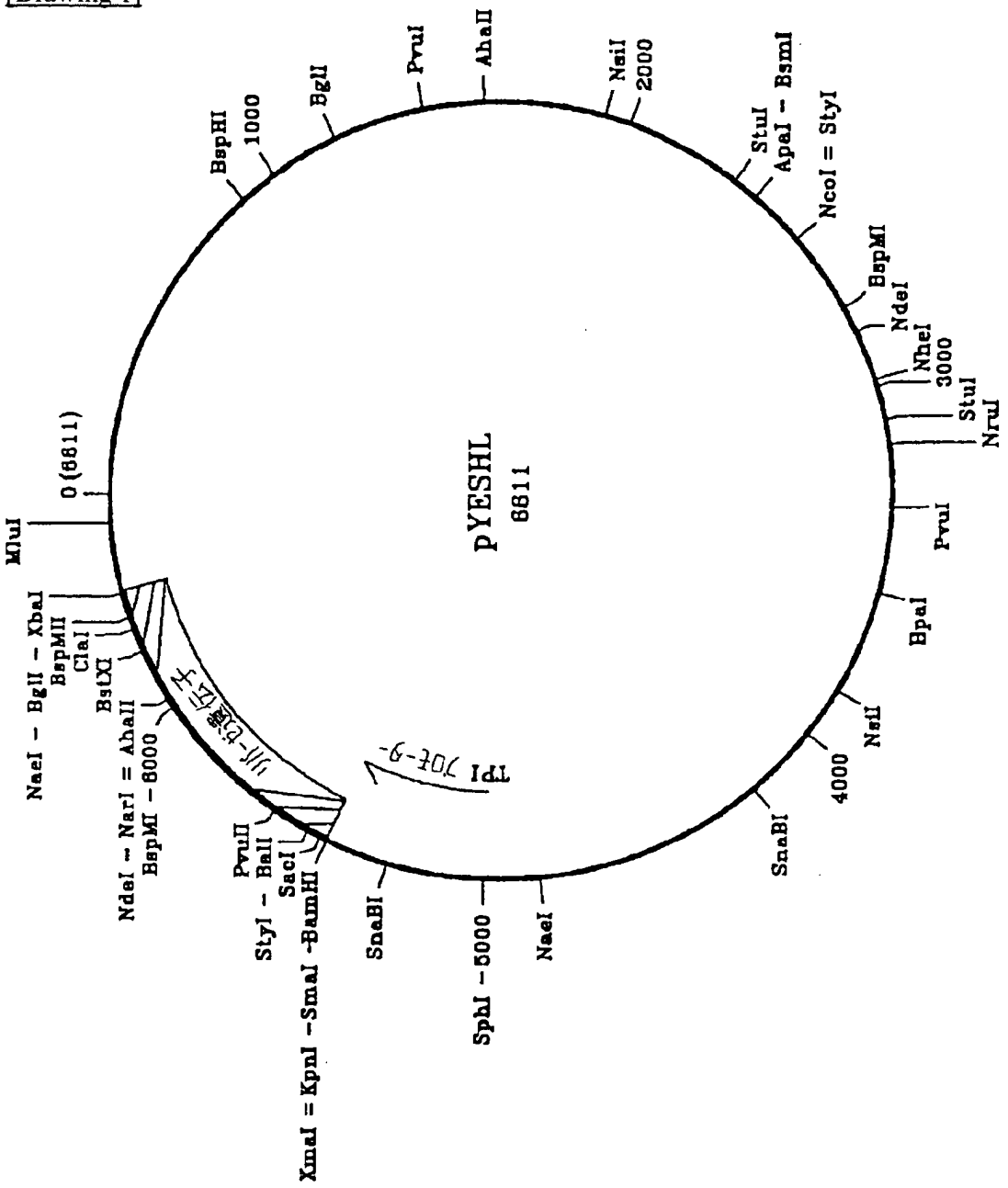

Fig. 1

[Drawing 2]

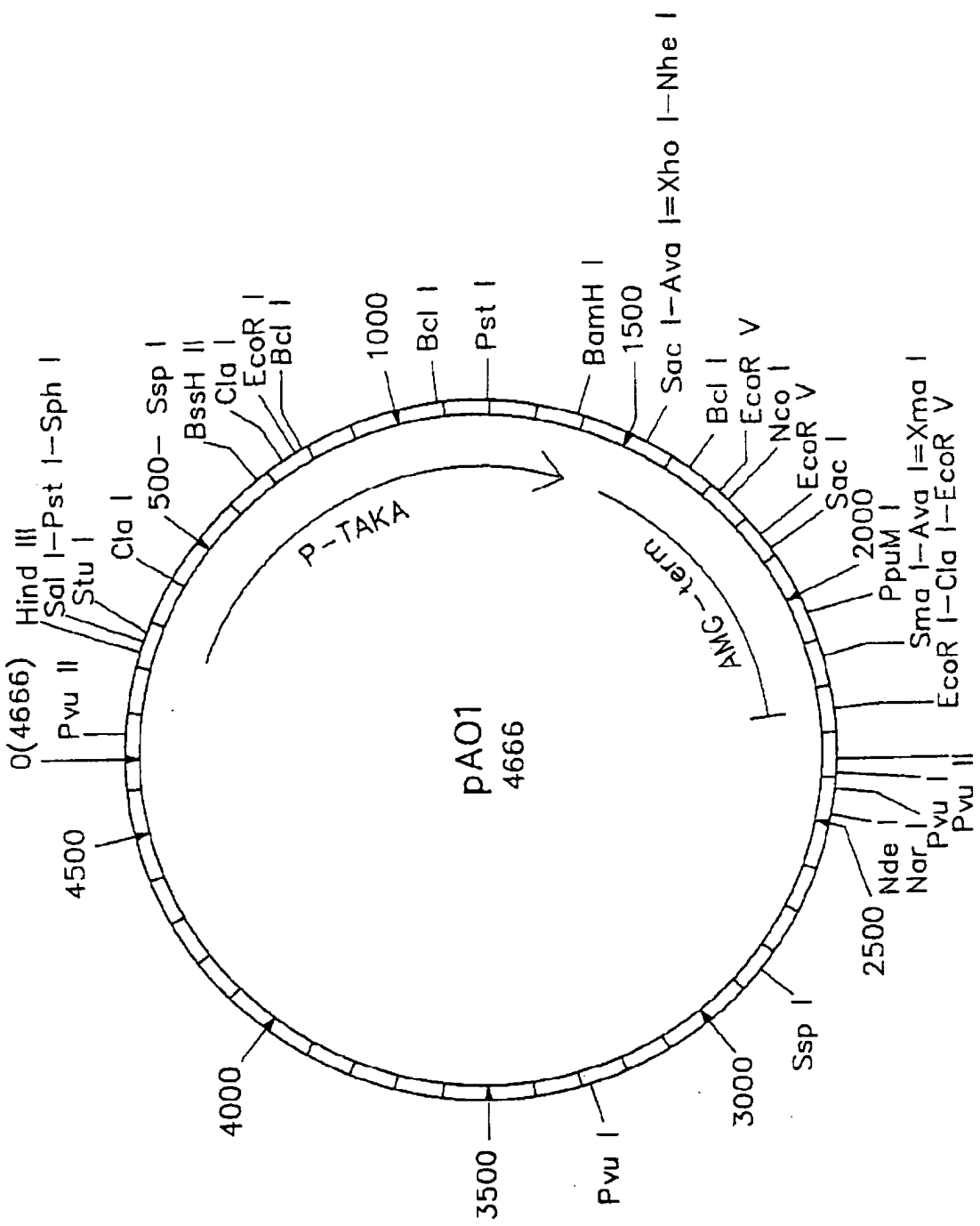

Fig. 2

[Drawing 3]

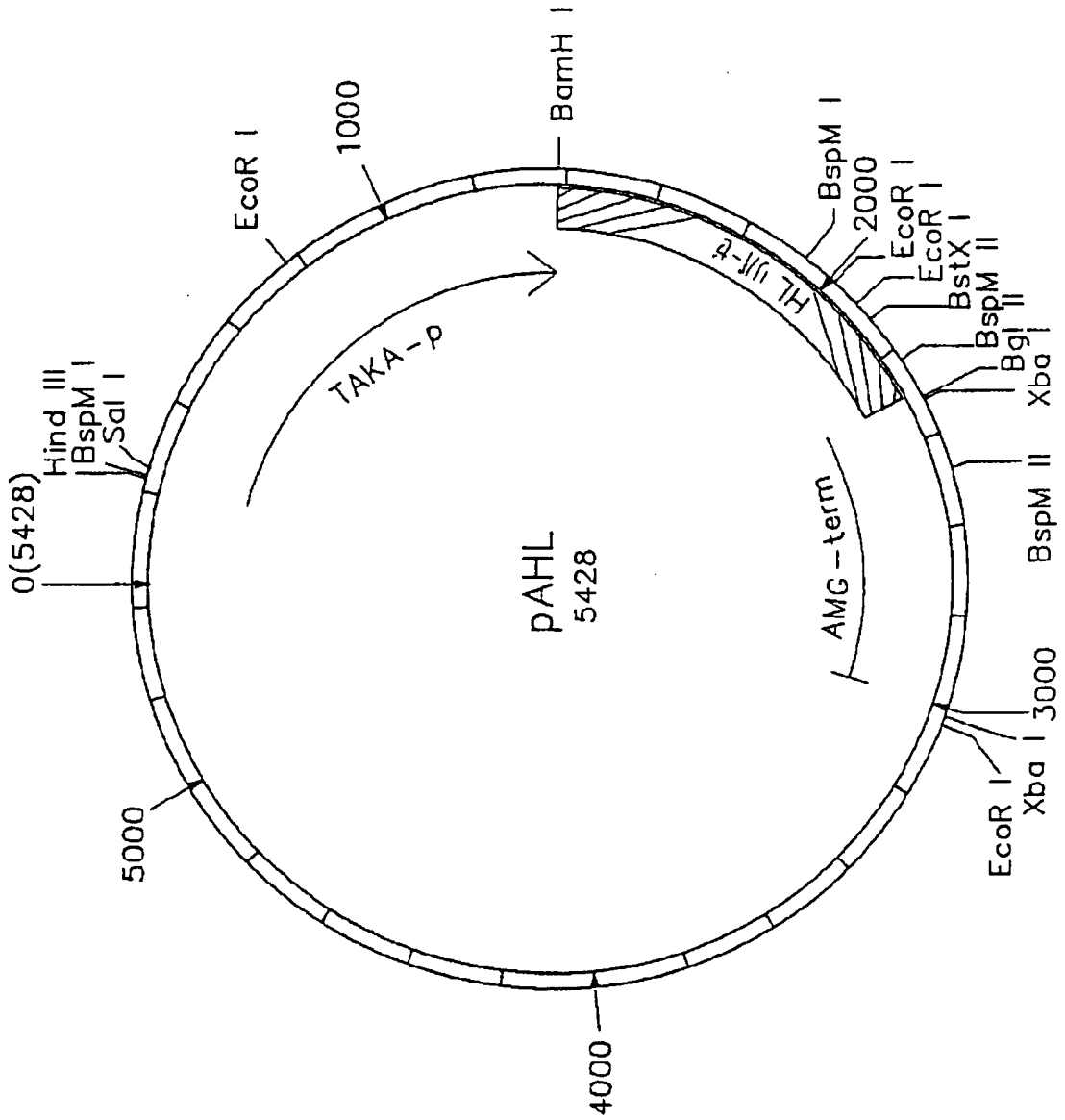

Fig. 3

[Drawing 4]

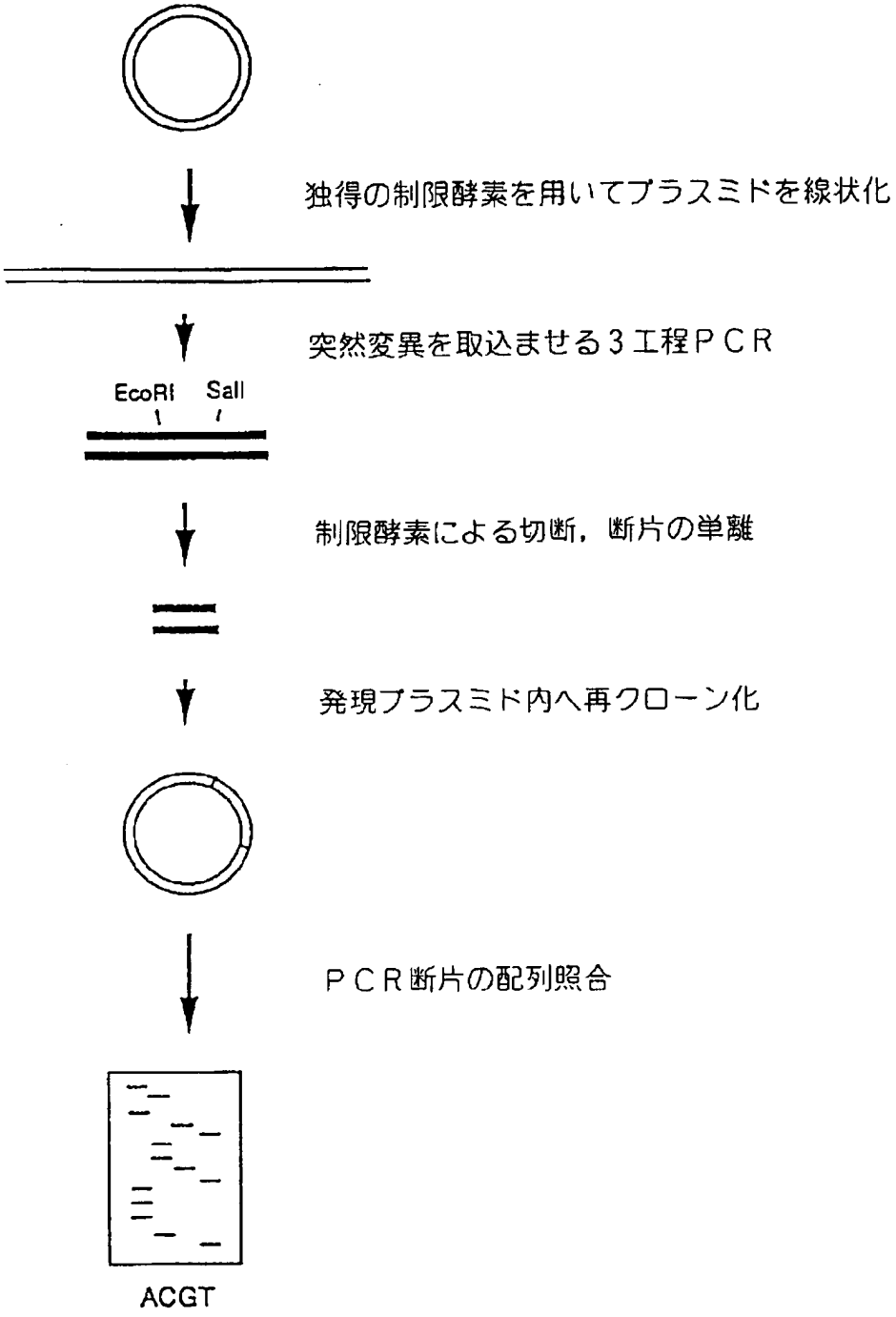
独得の制限酵素を用いてプラスミドを線状化
突然変異を取込ませる３工程ＰＣＲ
EcoRI
SalI
制限酵素による切断，断片の単離
発現プラスミド内へ再クローン化
ＰＣＲ断片の配列照合
ACGT

Fig. 4

[Drawing 5]

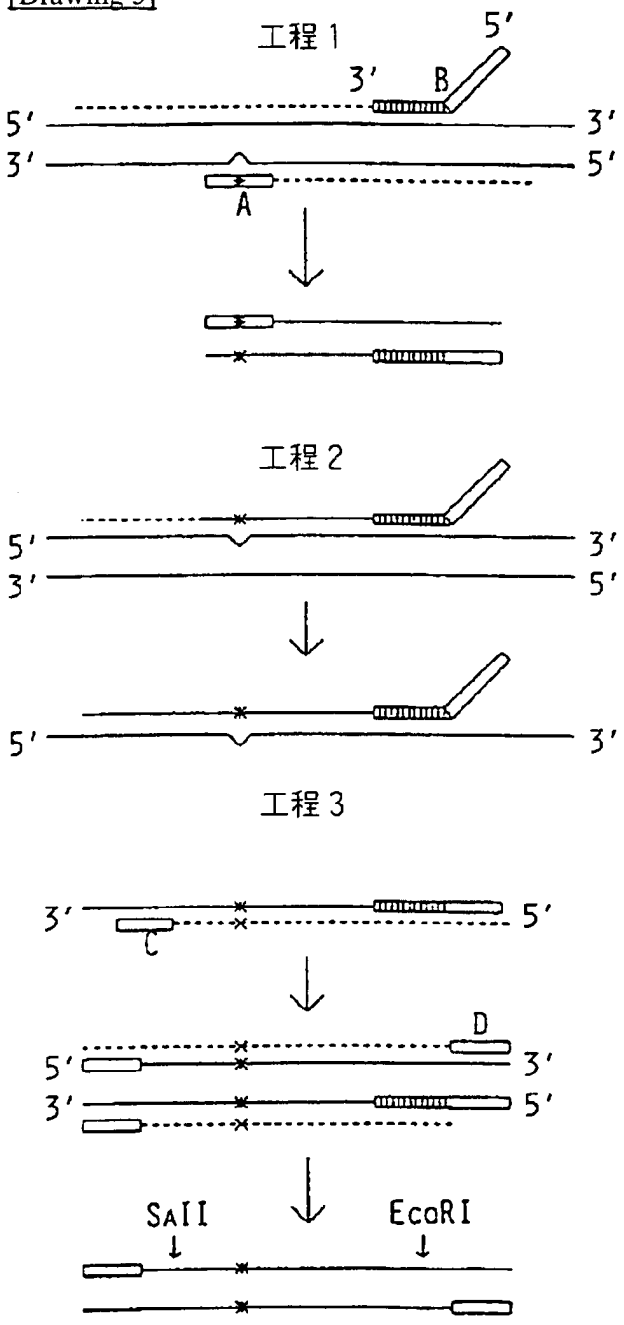

Fig. 5

WRITTEN AMENDMENT

[Procedure revision] 8 of Article 184 of Patent Law
[Filing Date] April 19, 1996
[Proposed Amendment]
CLAIMS
1. It is the Manufacture Approach of Variant of Parent Lipolytic Enzyme,

(a) Carry out the code of the parent lipolytic enzyme. A DNA array is left to the random mutagenesis,
(b) Mutagenesis obtained at the process (a) Discover a DNA array in a host cell and rank second.
(c) Mutagenesis lipid ****** which has the dependency made to decrease to calcium Said manufacture which comes to contain screening to the host cell which discovers base
Approach.
2. Further, Process (C) Compares with Parent Lipolytic Enzyme, and is ** to Detergent or Detergent Component.
The 1st term of a claim which comes to contain screening to the resistance by which the good was carried out
The approach of a publication.
3. Random Mutagenesis -- Use of Physical or Chemical-Mutagenesis-ized Agent -- Or use of an oligonucleotide -- or -- It is performed by use of the PCR creation mutagenesis.
A claim 1st or an approach given in dyadic.
4. Mutagenesis-ized Agent is Formic Acid, UV ****, Hydroxylamine, and N-Methyl. - N'
- Nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine,
A nitrous acid, an ethyl methane sulfonate (EMS), a sodium hydrogensulfite, and NUKU
The approach given in the 3rd term of a claim chosen from a REOCHIDO analog.
5. Mutagenesis About Manifestation of DNA Array, it is Mutagenesis. Suitable Host Cell Which Has DNA Array
By carrying out a transformation, it carries out and is the mutagenesis. A DNA array is the mutagenesis further by request. D
The code of the function to permit the manifestation of NA array is carried out. It comes to contain a DNA array and is ******.
** Host cell **** obtained at the process (b) under the suitable condition for discovering a DNA array
****** -- an approach given in the 1st term of a claim.
6. Mutagenesis Host Cell Used in order to Make DNA Array Discover is Microbial Cell.
The approach given in the 1st term of a claim which comes out and exists.
7. Direction of 6th Term Publication of Claim whose Host Cell is Cell of Fungus or Bacterial Strain
Law.
8. Host Cell is Group Aspergillus, for Example, A. Nigre, A. ORIZE, and A.
It is the cell of the cell of NIDORANSU, or group Saccharomyces, for example, S. SEREBISHIE, and is **.
An approach ** and given in the 7th term of a claim.
9. Host Cell is Gram Positive Bacterium, for Example, Bacillus. Subtilis, Bacillus Li KENIHORUMISU and Bacillus Wren -- TASS and Bacillus Brevis and Bacillus SUTEARO
Thermostat philus, Bacillus ARUKARO philus, Bacillus Friend RORIKUEFASHI ENSU, Bacillus A KOAGUYU lance, Bacillus A SAKYU lance, Bacillus RA UTASU, Bacillus CHU phosphorus diene cis- ** is a streptomyces. RIBIDANSU Or streptomyces They are MURINASU or a gram negative, for example, E. collie.
An approach given in the 7th term of a claim.

10. Mutagenesis Lipolytic Enzyme -- Non-Ion, Anion, Cation, and Zwitter Ion -- Moreover
The account of the 2nd term of a claim which has the resistance improved to **********
The approach of **.
11. Nonionic Surface Active Agent is Alcoholic Ethoxy Rate, and/or is A.
A NION surfactant Account of the 10th term of a claim which is LAS or alkyl SURUFATO
The approach of **.
12. About Host Cell Screened at Process (C), They are Second Mutagenesis Processing and **.
The claim 1st left to screening, re-isolation, and/or re-cloning
An approach given in a term.
13. Carry Out Code of the Parent Lipolytic Enzyme for Random Mutagenesis. It is Localization in a Part of DNA Array.
An approach given in any 1 term of the 1-12th changing terms of a claim.
14. Parent Lipolytic Enzyme is Lipase, Esterase, KURUCHINAZE, or HOSUHORIPA.
An approach given in any 1 term of the 1-13th terms of a claim which are - ZE.
15. Nature Dialytic Ferment of Lipophilic is Lipase, and Localization Random Mutagenesis is Parent RIPA.
the code of the lipid contact region of - ZE or its part is carried out **** about a part of DNA array
The approach of the breaking claim 13th or 14 term publication.
16. Localization Random Mutagenesis is Lid (Lid) Field and/or Non-dense of Parent Lipase.
Aquosity KUREFUTO (cleft) or this lid field, and/or a part of hydrophobic joint KUREFUTO
A code is carried out. Approach given in the 15th term of a claim performed about a part of DNA array
.
17. Parent Lipolytic Enzyme is not in the 1-16th Terms of Claim Which May Originate in Microorganism.
An approach given in ** or the 1st term.
18. The approach given in the 17th term of a claim a parent lipolytic enzyme may originate in a fungus.
19. Carry Out Code of the Parent Lipolytic Enzyme. DNA Array is Fumi Kola. Sp., RIZOMU Call S
p. -- Rhizopus sp. and Candida Approach given in the 18th term of a claim which may originate in sp.
.
20. Parent Lipolytic Enzyme is Lipase and Carry Out Code of the Parent Lipase. DNA Array
The strain of ** and H. RANUGINOSA, H. RANUGINOSA strain DSM 4109 [for example,], Rh. Mucor
The claim which is what may originate in ****** or the strain of C. anta RUKUCHIKA
An approach given in the 19th term.
21. Random Mutagenesis is Entrusted. DNA Array Can Obtain from DSM 4109.
** H. RANUGINOSA The amino acid residue 21-27 of lipase, 56-64, 81-99,108-116

145-147,174,202-213,226-227,246-259 or -- 263-269 Specification
The approach given in the 20th term of a claim which carries out the code of at least one of the **** fields.
22. ** to which Localization Random Mutagenesis is Performed in at Least Two of These Fields
The approach of ** given in the 20th term of the range.
23. The approach given in the 17th term of a claim a parent lipolytic enzyme may originate in bacteria.
24. Carry Out Code of the Parent Lipolytic Enzyme. DNA Array is Pseudomonas. Spp., for Example, P
. SEPASHIA, P. Alcaligenes, P. SHUDO Alcaligenes, or P. FURAGI
The 23rd term publication of a claim which can be obtained from the strain of a Bacillus from the strain of -
******.
25. H. RANUGINOSA Lipase Which Can be Obtained from DSM 4109, or Its Analog
Mutation [in / it is a variant and / at least one of the locations of a degree]: K46, E56, S58
,G61,T64,N73,S83,I90,G91,N92,N94,D96,L97,K98,E99,I100,D102
,A121,E129,D167,R205,E210,K237,N251,I252,D254,P256,G263,L264
Or said variant which comes to contain T267.
26. H. RANUGINOSA Lipase or this RIPA Which Can be Obtained from Strain DSM 4109
the variant of the analog of ZE -- it is -- amino acid residue 56-64 and 83-100 or -- 205-211 **
Said variant which has mutation in at least one of the fields pinpointed more.
27. The Following Mutation : K46R, D57G, S58F, G61S, D62C, T64R, S83T, I90F, G91A
,N92H,N94I,N94K,L97M,K98I,I100V,D102K,A121V,E129K,D167G,R205K
E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q, or T267W.
** -- the variant given in the 26th term of a claim which comes to contain one even if few.
28. The Following Mutation :
N94K+D96A

S83T+N94K+D96N

E87K+D96V

E87K+G91A+D96A

N94K+F95L+D96H

A121V+R205K+E210Q

F95C+D96N

G91S+L93V+F95C

E87K+G91A+D96R+I100V

E87K+G91A

S83T+E87K+Q249R

S83T+E87K+W89G+G91A+N94K+D96V

N73D+S85T+E87K+G91A+N94K+D94A

E87K+G91A+L93I+N94K+D96A

D167G+E210V

N73D+E87K+G91A+N94I+D96G

S83T+E87K+G91A+N92H+N94K+D96M

E210W

E56T+D57L+I90F+D96L+E99K

E56R+D57L+V60M+D62N+S83T+D96P+D102E

D57G+N94K+K96L+L97M

E87K+G91A+D96R+I100V+E129K+K237M+I252L+P256T+(

E56R+D57G+S58F+D62C+T64R+E87G+G91A+F95L+D96P+I

K46R,E56R,G61S

D102K

D167G

N73D+E87K+G91A+N94I+D96G

E210V

E210W

N251W+D254W+T267W

** -- H. RANUGINOSA which can be obtained from DSM 4109 which come to contain one even if few
Lipase or the variant of the analog.
29. To Dependency and/or Parent Lipolytic Enzyme Which were Made to Decrease to Calcium
Resistance **** which compared and was improved to the detergent or the detergent component
Mutagenesis which carries out the code of the variant of the lipolytic enzyme to carry out It comes to contain a DNA array. DNA construction
It is the body and **. Approach given [a DNA array] in any 1 term of the 1-23rd terms of a claim
The above isolated from the host cell screened in ****** (c) DNA
Construction object.
30. H. RANUGINOSA Lipase Given in Any 1 Term of the 24-28th Terms of Claim -- Strange
The code of the variant is carried out. DNA construction object.
31. A claim 29th or 30 term publication Vector which has a DNA construction object.
32. BEKU Given in 31st Term of Claim Which is Plasmid or Bacteriophage
TA.
33. Permit Manifestation of Variant of Parent Lipolytic Enzyme. Manifestation Which Comes to Contain DNA Array Further
The vector of the claim 31st or 32 term publication which is a vector.
34. Claim 29th or 30 Term Publication DNA Construction Object or the 31-33rd Terms of Claim are.
The host cell which has the vector of a publication in a gap or the 1st term.
35. The cell given in the 34th term of a claim which is a microbial cell.
36. The cell given in the 35th term of a claim which is the cell of a fungus or bacterial strain.
37. Group Aspergillus, for Example, A. Nigre, A. ORIZE, and A. Nidulans
The example of a claim which is the cell of ****** or group Saccharomyces, for example, S. cerevisiae
A cell given in **** 36 term.
38. Gram Positive Bacterium, for Example, Bacillus, Subtilis, Bacillus RIKENIHORUMISU
Bacillus Wren -- TASS and Bacillus Brevis and Bacillus SUTEARO thermostat filler SU, Bacillus ARUKARO philus, Bacillus Friend RORIKUEFASHIENSU, BASHI lath A KOAGYU lance and Bacillus A SAKYU lance and Bacillus Rau -- TASS and BASHI
Lath CHU phosphorus diene cis- ** is a streptomyces. RIBIDANSU or SUTOREPU TOMAISESU Claim which is MURINASU or a gram negative, for example, E. collie A cell given in the 36th term.
39. Dependency and/or Parent Lipolytic Enzyme to Calcium Which were Made to Decrease
It is ** about the variant of the parent lipolytic enzyme which compares and has the resistance over a detergent or a detergent component.
****** -- an approach -- it is -- an approach given in any 1 term of the 1-23rd terms of a claim -- ****
****** which manufactured the nature dialytic ferment of ****** body fat, and was

subsequently screened at the process (c)
Said approach of coming to contain collecting lipolytic enzymes from **.
40. Compare with Dependency and/or Parent Enzyme Which were Made to Decrease in Number to Calcium.
The variant of the parent lipolytic enzyme which has the resistance improved to the detergent or the detergent component is manufactured.
Bottom of suitable condition for being approach of carrying out and making variant discover, and claim the 34-
It is a culture about the variant which cultivated the host cell of a publication in any 1 term of the 38th term, and was subsequently discovered.
since -- said approach of coming to contain collecting.
41. Be in Form of Non-Dust Nature Granulated Material, Stabilizer Object, or Protected Enzyme by Request,
the variant of the lipolytic enzyme of a publication is included in any 1 term of the 24-28th terms of a claim
The additive for ******.
42. 0.02-200Mg per 1G of Additives Claim Containing Enzyme Protein
The additive for detergents given in the 41st term.
43. They are Other Enzymes, for Example, Protease, Amylase, and Peroxidase Additionally.
The claim 41st which comes to contain KUCHINAZE, lipase, and/or a cellulase
Or the additive for detergents given in 42 terms.
44. It is ** about Variant of Lipolytic Enzyme Given in Any 1 Term of the 24-28th Terms of Claim.
The detergent constituent which becomes by **.
45. They are Other Enzymes, for Example, Protease, Amylase, and Peroxidase Additionally,
The 44th term of a claim which comes to contain KUCHINAZE, lipase, and/or a cellulase
The detergent constituent of a publication.

CORRECTION OR AMENDMENT

[Kind of official gazette] Printing of amendment by the 1st term of Article 17 of Patent Law, and the convention of 2 of Article 17 of Patent Law
[Section partition] The 1st partition of the 1st section
[Publication date] May 21, Heisei 14 (2002. 5.21)

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C12R　1:69　)
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C11D　3/386
7/42
C12N　1/19
1/21
9/20

手 続 補 正 書

平成１３年１２月３日

特許庁長官　及 川 耕 造 殿

1．事件の表示

平成７年特許願第５２１５２５号

2．補正をする者

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4．補正対象書類名

請求の範囲

5．補正対象項目名

請求の範囲

6．補正の内容

請求の範囲を別紙の通りに補正します。

7．添付書類の目録

請求の範囲　　１通

請求の範囲

1．親リパーゼの変異体の製造方法であって、

（a）親リパーゼをコードするDNAをランダム変異誘発に委ね、

（b）工程（a）で得られた変異誘発処理されたDNAを宿主細胞中で発現せしめ、そして

（c）カルシウムに対して低下した依存性を有する変異したリパーゼを発現する宿主細胞についてスクリーニングする、

ことを含んで成る方法。

2．工程（c）が更に、親リパーゼと比較して洗剤又は洗剤成分に対して改善された耐性を有する事についてスクリーニングすることを含んで成る、請求項1に記載の方法。

3．前記リパーゼがフミコラ（Humicola）属の株から得られうる、請求項1又は2に記載の方法。

4．前記リパーゼがフミコラ．ラヌギノサ（Humicola lanuginosa）DSM 4109株から得られうる、請求項3に記載の方法。

5．フミコラ．ラヌギノサDSM 4109から得られうるリパーゼの変異体であって、次の変異：S83T、G91A、I100V、D167G、の少なくとも1つを含んでなり、場合によっては更に、リパーゼのN末端及びC末端の一方又は両方への1又は複数のアミノ酸残基の付加、アミノ酸配列中の1又は複数の異なる部位における1又は複数のアミノ酸残基の置換、アミノ酸配列中の1若しくは複数の部位又はリパーゼの両端若しくは一端における1又は複数のアミノ酸残基の除去、あるいはアミノ酸配列中の1又は複数の部位における1又は複数のアミノ酸残基の挿入を含んで成る、リパーゼの変異体。

6．フミコラ．ラヌギノサDSM 4109から得られうるリパーゼの変異体であって、次の変異：

D94K+D96A

S83T+D94K+D96N

E87K+D96V

E87K+G91A+D96A

N94K+F95L+D96H

F95C+D96A

E87K+G91A+D96R+T110V

E87K+G91A

S83T+E87K+Q249R

S83T+E87K+W89G+G91A+N94K+D96V

N73D+S83T+E87K+G91A+N94K+D96A

E87K+G91A+L93T+N94K+D96A

D167G E210V

N73D+E87K+G91A+N94I+D96G

S83T+E87K+G91A+N92H+N94K+D96M

D56R+D57L+V60M+D62N+S83T+D96P+D102E

D57G+N94K+K96L+L97M

E87K+G91A+D96R+T110V+E129K+K237M+I252L+P256T+G265A+L264Q

D56R+D57G+S58F+D62C+T84R+E87G+G91A+F95L+D96P+K98I+K237M

D167G

N73D+E87K+G91A+N94I+D96G

S83T+E87K+G91A+N92H+N94K+D96M

D57G+N94K+D96L+L97M

G91A+N94K+D96A,

の少なくとも１つを含んでなり、場合によっては更に、リパーゼのN-末端及びC-末端の一方又は両方への１又は複数のアミノ酸残基の付加、アミノ酸配列中の１又は複数の異なる部位における１又は複数のアミノ酸残基の置換、アミノ酸配列中の１若しくは複数の部位又はリパーゼの両端若しくは一端における１又は複数のアミノ酸残基の除去、あるいはアミノ酸配列中の１又は複数の部位における１又は複数のアミノ酸残基の挿入を含んで成る、リパーゼの変異体。

７．請求項５又は６に記載のリパーゼ変異体をコードするDNA構成物。

８．請求項７に記載のDNA構成物を含む宿主細胞。

９．リパーゼ変異体の製造方法において、該変異体を発現するのに適当な条件

下で請求項8に記載の宿主細胞を培養し、そして発現した変異体を培養物から回収することを含んで成る方法。

１０．請求項５又は６に記載のリバーゼ変異体を含んで成る洗剤組成物。